Caractérisation de transporteurs membranaires de Plasmodium falciparum en tant que potentiel cibles thérapeutiques

Stéphanie Bosne

UNIVERSITÉ PARIS-SUD

ÉCOLE DOCTORALE 419 :
BIOSIGNE

Laboratoire : *UMR 8221 - Laboratoire des Protéines Membranaires (CEA Saclay)*

THÈSE

SCIENCES DE LA VIE ET DE LA SANTÉ

par

Stéphanie CORREIA DE MATOS DAVID BOSNE

Characterization of *Plasmodium falciparum* membrane transporters as potential antimalarial targets

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Composition du jury :

Directeur de thèse :	Christine JAXEL	Chercheur CNRS, UMR 8221 CNRS, CEA SACLAY
Co-directeur de thèse :	Marc le MAIRE	Professeur, Université Paris-Sud
Président du jury :	Oliver NÜSSE	Professeur, Université Paris-Sud
Rapporteurs :	Bruno MIROUX	Directeur de Recherches INSERM, IBPC, Paris
	Martin PICARD	Chercheur CNRS, CNRS UMR 8015, Paris
Examinateurs :	Isabelle FLORENT	Professeur MNHN, MNHN Paris
	Philippe LOISEAU	Professeur, Université Paris-Sud

Merci à tous ceux qui ont rendu cette thèse possible,
rien n'aurait été réalisable sans vous !

A minha Mãe, à Alexandre,

Pour avoir été présent dans les meilleurs et les pires moments, merci !

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Introduction

I – Malaria

Malaria is a vector-borne infectious disease caused by a parasite of the *Plasmodium* genus. It is commonly transmitted by a bite from an infected female *Anopheles* mosquito, which introduces the parasites in the blood stream of a human host. Malaria is a life-threatening disease and half of the world population is at risk of having malaria (World Health Organization, 2013a).

Malaria is endemic from 99 countries in Central and South-America, Sub-Saharan Africa and South-East Asia (Heppner, 2013; World Health Organization, 2013b) (Figure 1). According to the World Health Organization (WHO) in 2012 there was an estimated of 627 000 malaria related deaths, from about 207 million infected people (World Health Organization, 2013b).

I.1 – Historical and Sociological Context of Malaria

I.1.1 – The socio-economic burden of malaria

Malaria is commonly associated with poverty but it is also a cause of poverty, being an obstacle for economic development. Although it is endemic to tropical regions, it affects travelers from developed countries, affecting people worldwide, beyond borders. It has been pointed to be the major factor for the lag on economic growth retarder in South America. Poverty also increases also the risk of malaria. It is a vicious cycle. In some countries the burden of the disease represents up to 40% of public health spending (http://www.rollbackmalaria.org/index.html). Poverty, political issues, population migration, poor supply and high counterfeit of antimalarial drugs (up to 40% of artesunate-based malaria medications are counterfeited in Asia (Parry, 2005)), heterogeneous access to quality health installations, cultural and religious beliefs, all of these are major factors that contribute to the burden of the disease and to the difficulty of malaria eradication, especially in Africa (Heppner, 2013).

Malaria most severely affects young children and women in their first pregnancy. The major public health problems are: i) the long term cognitive damage in children attained by cerebral malaria, being one of the leading causes of cognitive and developmental disabilities, which largely contributes to the burden of malaria in poor countries (Idro et al., 2005); ii) dual-infection of HIV and malaria (Abu-Raddad et al., 2006; Heppner, 2013); iii) the increased susceptibility to other diseases; iv) and malaria during pregnancy, with lifelong affects to the unborn child (Umbers et al., 2011). Preventing placental malaria in first pregnancy women would prevent one of the great causes of malaria related death and birth of low weight children in sub-Saharan Africa.

I.1.2 – A long story made short

The term *Malaria* originates from medieval Italian and means *mala aria* or *maus ares,* meaning “bad air”. Probably arising from the association of the disease to swamps and marshlands, which we now know are the breading grounds of the mosquitos transmitting the parasites (Reiter, 2000).

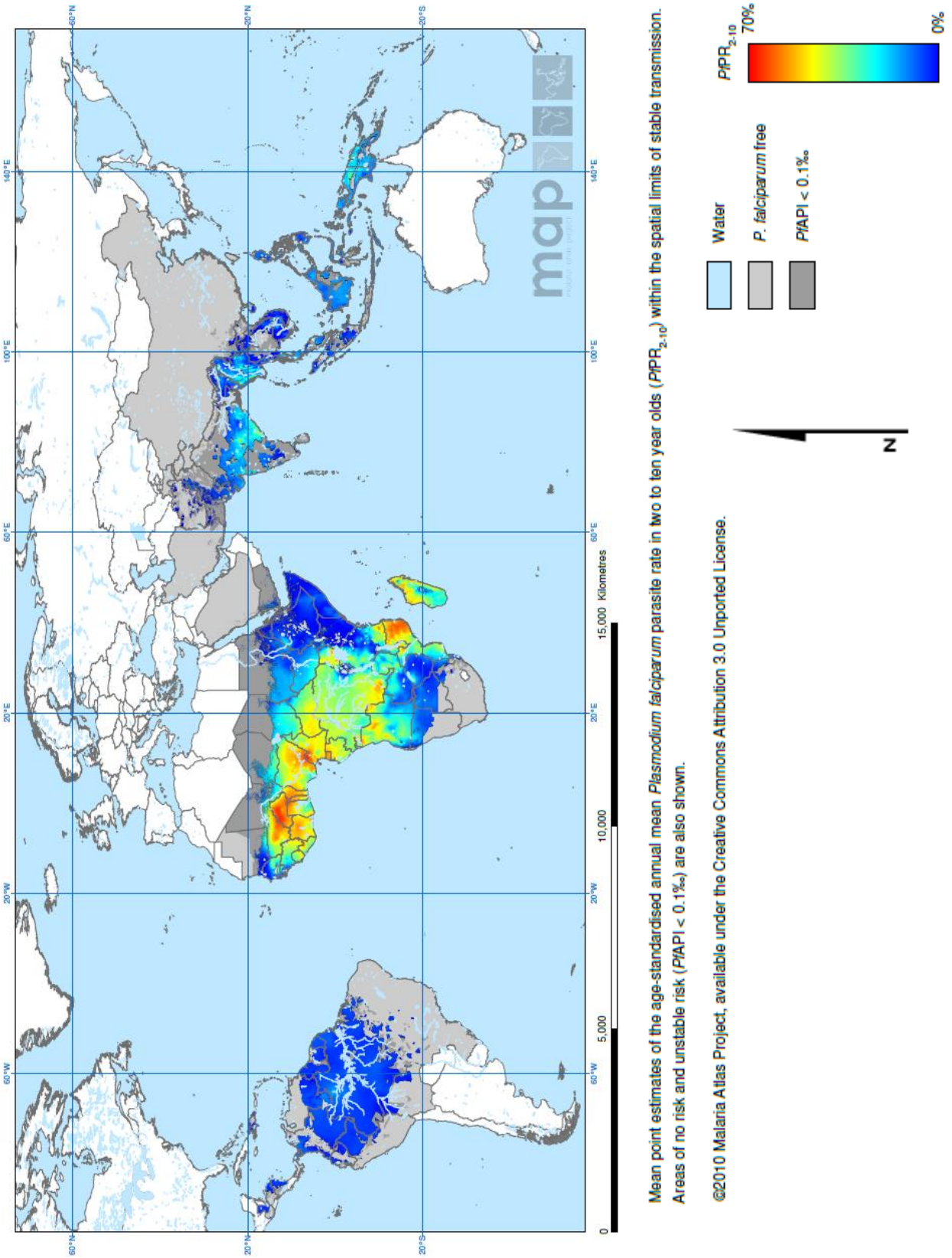

Figure 1 – Spatial distribuition of Malaria endemecity areas in 2010.

Malaria Atlas Project (MAP): http://www.map.ox.ac.uk/ (Gething et al., 2011a)

I.1.2.1 - The first reports of the disease

Some data suggest that the *P. falciparum* malaria may have evolved from a gorilla parasite (Prugnolle et al., 2011). Despite the fact that the parasite responsible for malaria has been on earth for 50.000 – 100.000 years, the first references of malaria were in 2700 BC in ancient China (Cox, 2002), but the first evidence of malaria comes from Egyptian mummies of over 5200 years old. The Egyptians also described malaria signs in ancient treaties like the Ebers papyrus, 1500 BC (Cox, 2002). Hippocrates also described clinical signs of malaria, especially fevers, in 700 BC. Through history there exist references to these "periodic fevers". Malaria was once present in Europe and North America (Figure 3) (Lindemmann, 1999). It was also known as the "Roman Fever" as it could have been at the origin of the decline of the Roman Empire. Malaria dramatically increased due to world population growth and is linked to the development of agriculture and densification of population when the first cities appeared. Agricultural techniques favored mosquito proliferation by increasing breading sites through irrigation runoff and drainage problems.

I.1.2.2 - The first scientific studies

The first important advances in malaria scientific discoveries were made in 1880. Charles Louis Alphonse Laveran was a French doctor of a military hospital in Algeria that observed for the first time parasites in red-blood-cells of an infected patient, and lack of parasites in healthy people. He also observed that the parasites disappeared with the use of quinine (Laveran, 1880). He proposed that this organism was responsible for causing malaria. Laveran called this parasite *Oscillaria malariae, a posteriori* it received the common name of Laveran's germ. One year later a Cuban doctor, Carlos Finlay, brought strong evidences that mosquitoes were responsible for transmitting the disease, confirming previous suggestions of Josiah C. Nott and Patrick Manson (Tan and Sung, 2008). In 1885 Ettore Marchiafava and Angelo Celli confirmed Laveran's findings by observing, with an oil immersion microscope, amoeboid parasites in individuals affected by "swamp fever" which they called *Plasmodium.* Golgi in 1886, and then Marchiafava and Celli in 1889 described 3 of the 5 *Plasmodium* species infecting humans.

The Scottish doctor Sir Ronald Ross described the complete life cycle of the malaria parasite in the mosquito, when working in a hospital of Calcutta (India). In 1897, he dissected infected mosquitoes, and proved that *Anopheles* was the transmiting vector of malaria to humans. Subsequently he led campaigns for the control of malaria. In 1900 the findings of Sir Ronald Ross and Carlos Finlay were confirmed by a medical committee chaired by Walter Reed, and Ross's recommendations were implemented in the construction of the Panamá channel, saving thousands of lives and helped to define public health strategies to fight against the disease (Simmons, 1979). In 1902, Sir Ronald Ross received the Nobel price of Medicine. He went on to lead campaigns for the control of malaria. In 1907 Louis Alphonse Laveran also received the Nobel Price of Medicine for his early work on Malaria.

I.1.2.3 – The War

Through history Malaria took an important toll on civilizations. In 1910, Sir Ronald Ross, in his book *Prevention of Malaria* included a chapter entitled "The Prevention of Malaria in War."

During World War I, Cinchona bark and quinine were widely used. The supplies were not enough to cover the needs, in particular because of destroyed cinchona's plantations. Since then, a lot of financial investments went into new antimalarial research. During World War II and the Vietnam War, malaria was the most important health hazard for the U.S army in South Pacific, infecting 500.000 men (Bray, 2004). In 1942, the U.S.A established the Malaria Control in War Areas (MCWA), and its successor, the Communicable Disease Center, nowadays the Center for Disease Control and Prevention, also known as CDC, was established in 1946. The first chloroquine was synthesized in 1934 by Bayer laboratories, although it was only a decade later that it was commercialized due to its high toxicity (Trager and Jensen, 2005).

I.2 - Plasmodium

I.2.1 – *Plasmodium* parasites

Plasmodium is a large genus of eukaryote unicellular parasites, also called protozoa. Five *Plasmodium* species can infect humans:

- *Plasmodium falciparum* is the most frequent species infecting humans and is responsible for the most severe form of malaria, the malignant tertian malaria (recrudescent fevers every 2 days). This form of malaria is characterized by severe anemia and can lead to cerebral malaria, a complication that can be fatal. *Plasmodium falciparum* can be maintained in culture with human erythrocytes. The 23-megabase nuclear genome organized in 14 chromosomes, encodes for about 5,300 genes, and is the most (A/T)-rich genome sequenced to date (Gardner et al., 2002).

- *P. vivax* (typically found in Asia, Latin America, and in some regions of Africa) and *P. ovale* (mostly found in Africa and the islands of the western Pacific Ocean) causes a benign form of malaria, the benign tertian malaria. These species have the particularity of developing dormant liver stages ("hypnozoite"). These can relapse several months or even years after the infection has occurred and the disease can become chronic. These people constitute reservoirs of parasites in endemic regions but can also introduce the disease in malaria-free regions. *P. vivax* is the most widespread species and is responsible for the most prevalent form of malaria it is rarely fatal, but it remains a great cause of morbidity, and thus it is considered a neglected disease (Grellier et al., 2012).

- *P. malariae* (found worldwide) is less frequent and causes the benign quartan malaria (three days fever cycle). When untreated it can cause a long-lasting, chronic infection that can last a lifetime and cause serious complications. *Plasmodium knowlesi* is much less frequent and was recently discovered to infect humans in Malaysia (White, 2008a).

The only known host of *P. falciparum* and *P. malariae* are humans. The other species are zoonotic[1] and thus have other animal hosts, such as primate which constitutes a reservoir for the parasites.

I.2.2 – *Plasmodium falciparum* life cycle

The *Plasmodium falciparum* life cycle involves two hosts: a female mosquito vector of *Anopheles* genus and a Human (Figure 2). Over 30 Anopheles mosquito species are known to transmit human malaria.

A- Infection and Human liver stages - exo-erythrocytic schizogony

P. falciparum lives in the gut and salivary glands of the female *Anopheles* mosquitoes. During a blood meal the infected mosquito injects the sporozoites present in the saliva, into the bloodstream of a human (see 1 in Figure 2). Sporozoites migrate and infect hepatocytes (see 2 in Figure 2) where they multiply asexually (5 – 16 days) and mature into schizonts, structure that contains thousands of merozoites (see 3 in Figure 2). During this period no illness is caused (Khan and Lai, 1999). After rupture of the liver cell, the merozoites (see 4 in Figure 2) are free to infect erythrocytes (see 5 in Figure 2) and undergo asexual erythrocytic cycle (see B in Figure 2).

B- Erythrocytic Cycle - erythrocytic schizogony

Each merozoite invades an erythrocyte within 1-2 minutes, consumes the hemoglobin for energy, and becomes an immature trophozoite (ring stage). After maturation and multiplication it differentiates into a schizont (see 6 in Figure 2). The schizonts rupture frees new merozoites that will infect other red blood cells (see 5 in Figure 2) and reiterate the asexual erythrocytic cycle (see B in Figure 2) (every 1 – 3 days). Trophozoits can also differentiate into sexual forms and originate gametocytes (see 7 in Figure 2). The rupture of red blood cells, which liberates merozoites, is responsible for the clinical manifestation of malaria.

C- Sporogonic Cycle - sexual stage

Some of the blood-stage merozoites do not develop into schizonts, but rather differentiate into male and female gametocytes (see 7 in Figure 2). These can be ingested by a female mosquito during a blood meal (see 8 in Figure 2), perpetuating the cycle. The male gametocyte (microgametocytes) and the female gametocyte (macrogametocytes), fertilize in the mosquito's stomach, producing zygotes (see 9 in Figure 2). The zygotes develop to become elongated - motile ookinetes (see 10 in Figure 2). These penetrate into the mosquito's mid-gut wall and differentiate into oocysts (see 11 in Figure 2). The oocysts grow, divide and, after 8 – 15 days the oocysts rupture releasing sporozoites (see 12 in Figure 2) that will migrate into the mosquito's salivary glands.

When this infected *Anopheles* female mosquito takes another blood meal, it will inoculate the sporozoites present in its salivary glands into another human and the cycle starts all over again. The mosquito acts as a vector, carrying the parasite from a human to another, and is not affected by the *Plasmodium* infection.

[1] Zoonoses- are infectious diseases that are transmited from a non-human species to a human or vice-versa, with or without an intermediate vector.

2 Parasitemia is a measure of the quantity of parasite present in the blood, reflecting the medical condition of the infection. It can be either expressed in percentage (%) of infected erythrocytes in thin smears of blood, in relation to a enumerated quantity of erythrocytes; or in

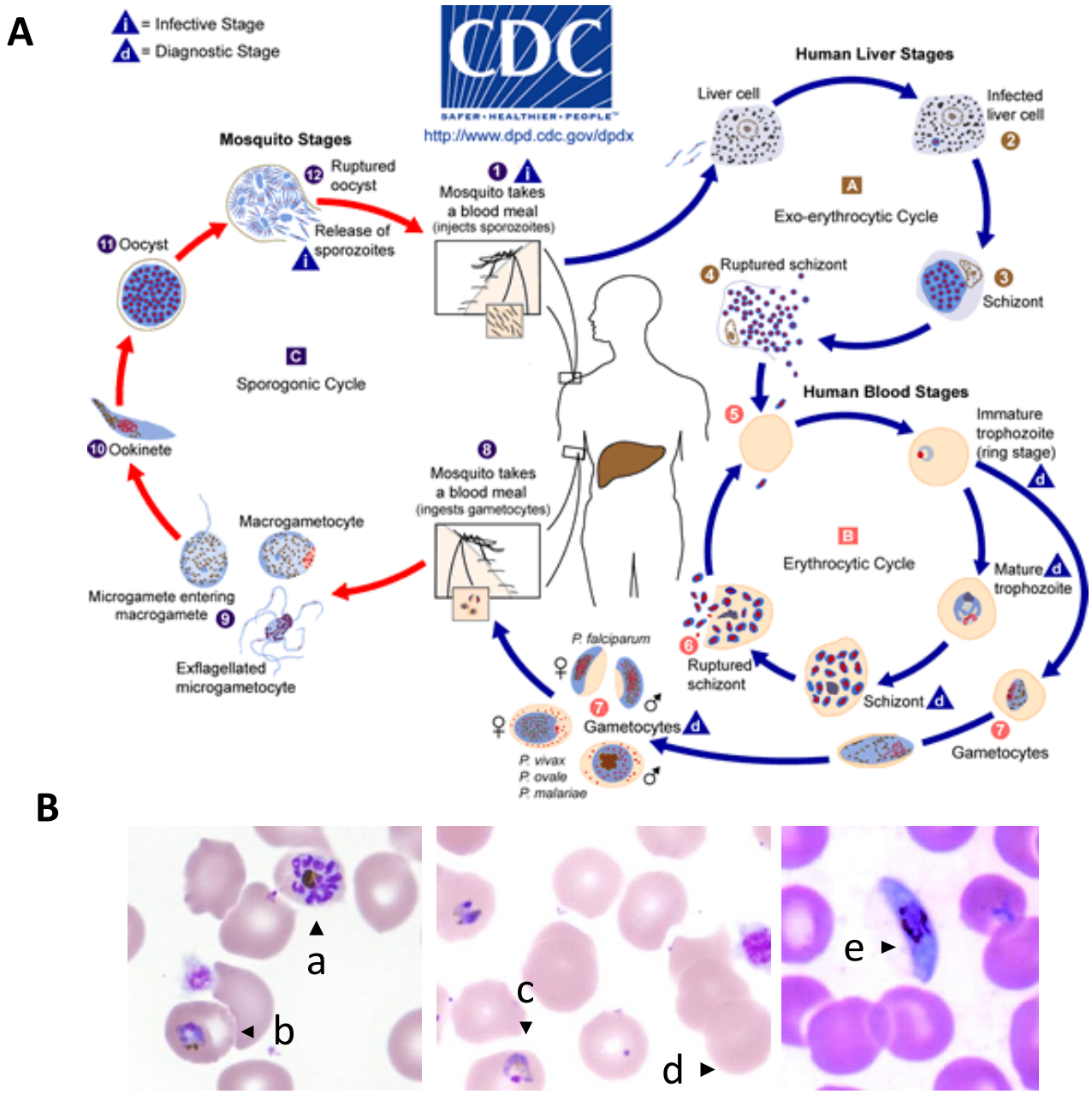

Figure 2 - Plasmodium falciparum life cycle. A) Schematic representation of P. falciparum life cycle; B) blood smear of infected patients colored with Giemsa.

A) ❶ sporozoites inoculation; A exo-erythrocytic cycle; ❷ infected liver cells; ❸ schizonts; ❹ rupture of schizont, merozoites liberation; B erythrocytic cycle; ❺ red blood cell infection; ❻ schizonts rupture releasing merozoites; ❼ gametocytes (sexual erythrocytic stages); C sporogonic cycle (parasite multiplication in the mosquito); ❽ mosquito infection during blood meal; ❾ male (microgametocytes) and female (macrogametocytes) gametocytes; ❿ ookinetes; ⓫ oocysts, ⓬ oocyst rupture and sporozoites releasing; i infective stage; d diagnostic stages. B) a – schizont; b – mature trophozoite; c – ring stage (immature trophozoite); d – non-infected erythrocyte; e - gametocyte. Source: Centers for Disease Control and Prevention – CDC, American Society of Hematology, CMAJ. (CDC, NIH, http://www.niaid.nih.gov/topics/malaria/pages/lifecycle.aspx, http://www.cdc.gov/malaria/about/biology/)

I.3 – The Disease: Prevention and Treatments

I.3.1 – The Symptoms

The liver stages of *Plasmodium* replication are asymptomatic. Malaria manifests during the erythrocytic cycle of the parasite. The symptoms are: generalized fatigue, loss of appetite, dizziness, intense headaches, digestive problems (upset stomach), nausea, vomiting, joint pain, abdominal pain, diarrhea, diffuse muscle pain. The clinical signs are characterized by: anemia, cyclic violent fevers with intermittent shivers (every 48h, 72h for *P. malariae infections*, excessive perspiration, convulsions, hemoglobinuria (blood in urine), jaundice, hypoglycemia, splenomegaly and hepatomegaly (spleen and liver enlargement, respectively). Many of these symptoms are non-specific and misdiagnosis becomes a major problem in malaria treatment.

The severe form of malaria appears 6 – 14 days after infection and is almost exclusively due to a *P. falciparum* infection (Trampuz et al., 2003). This form of the disease can lead to coma and death. Pregnant women and under 5 years old children are the most vulnerable. It starts with severe headaches and then cerebral ischemia takes place. In children, the cerebral malaria is characterized by an abnormal posture with the spinal cord in a backwards arching position (opisthotonus position). Severe malaria can progress very fast, in a few days or even hours (Trampuz et al., 2003). A rapid and reliable diagnosis is extremely important to avoid these cases.

1.3.2 – Diagnosis

In many remote malaria endemic regions a simple laboratory test is not possible. Fever periodicity is often used as a diagnosis, although this method is fallible. In endemic regions were antimalarials are widely used either in prophylaxis or for malaria treatment, the evolution of the symptoms is not the same as described above, frequently the rhythmic violent fever periods are absent being rather a gradual fever increase. Malaria is frequently misdiagnosed especially in children, leading to non-adapted treatment.

The most efficient and cheapest diagnosis method is still the microscope observation of a blood smear or a thick blood drop. The advantage of using a thick blood drop is the analysis of a larger amount of blood, increasing the probability of detecting very low parasitemia [2] (down to 0.0001% parasitemia or 5-20 parasites / µl of blood). This technique is often allied to a Giemsa coloring technique (Figure 2 B). This enables a full diagnosis of the disease, by identifying the *Plasmodium* species causing the infection (Warhurst and Williams, 1996) [3].

2 Parasitemia is a measure of the quantity of parasite present in the blood, reflecting the medical condition of the infection. It can be either expressed in percentage (%) of infected erythrocytes in thin smears of blood, in relation to a enumerated quantity of erythrocytes; or in number of parasites found per µl of a thick drop of blood, in relation to a standard number of red blood cells.

3 Giemsa is a coloring technique first developed by Gustav Giemsa in 1902. It specifically colors chromosomes in a purplish pink with methylene blue and eosin. By visualization of the nucleus of the parasite in the enucleated erythrocyte, parasitemia can be counted.

However a microscope and trained medical staff is needed to perform Giemsa technique. In field dispensaries, far from a health structure, it is often not the case. For field diagnosis, rapid diagnosis tests were developed (RDT) (see Box 1) (Abba et al., 2011; Kattenberg et al., 2011). RDT are conceived to be simple to use, with little training required of the health care staff and no special infrastructure needed. These tests are delivered as cassettes, reactive bands or dipsticks and they need only a drop of blood (Pattanasin et al., 2003). They are based on the immune-detection of an antigen from the parasite coupled to a chromatographic reaction. They usually take 15 – 20 minutes to detect the presence of the parasite in the blood sample. The limit of detection of this test is of 100 parasites per µl of blood, whilst a microscope can detect up to 5 parasites by µl of blood.

Box 1 | **Rapid Diagnosis Tests**
The first RTDs developed detected the parasite glutamate deshydrogenase (PGluDH) (Ling et al., 1986), but were replaced by the detection of the lactate deshydrogenase (PLDH). This last enzyme is essential for the generation of ATP during the glycolytic cycle, and hence is one of the most abundant. The presence of this enzyme is narrowly related to a *P. falciparum* infection. Nowadays there exist numerous RDTs with different specificities and sensibilities, detecting different species of *Plasmodium* (Ashley et al., 2009; Moody, 2002).

If a laboratory is available, molecular methods are also possible. By real-time PCR very low parasitemia can be detected (down to 1 parasite per µl of blood) (Mens et al., 2010). This technique is one of the most expensive and requires special conditions, a laboratory equipped with a PCR machine, well trained personnel, and respect of the cold chain during transport and storage of the blood sample. The major disadvantage of these two techniques is that they only show the level of parasitemia of the person, and not the progression of the disease (presence of early or mature stages or even gametocytes); for this the microscopic techniques are very useful.

I.3.3 – Prevention

Malaria prevention measures are the most valuable way to control the spread of the disease. These measures caused a drastic drop of new malaria cases since the beginning of the 20th century, when malaria was present in southern Europe and the United States (Figure 3). Vector control programs largely contributed to this eradication: prohibition of the inundation of agriculture grounds; draining stagnant water, amelioration of sanitation and housing (use of glass windows).

With the new prevention measures implemented by WHO, malaria mortality has been drastically decreasing in the world by 42% since 2000. The WHO prevention goals are: i) the coverage of 100 % of the population with long lasting insecticide-treated nets (ITN), ii) indoor residual spraying (IRS) (see Box 2), iii) protection of individuals by insect repellents and insect repellent clothing, iv) malaria prophylaxis for travelers, v) and vaccine research, along with the regular control of the population and providing treatment to infected people. However, each method has to be adapted to the region

under control, for instance, in sub-Saharan Africa some mosquitoes start feeding early in the evening, and in south Asia the vectors prefer being outdoors rather than indoors.

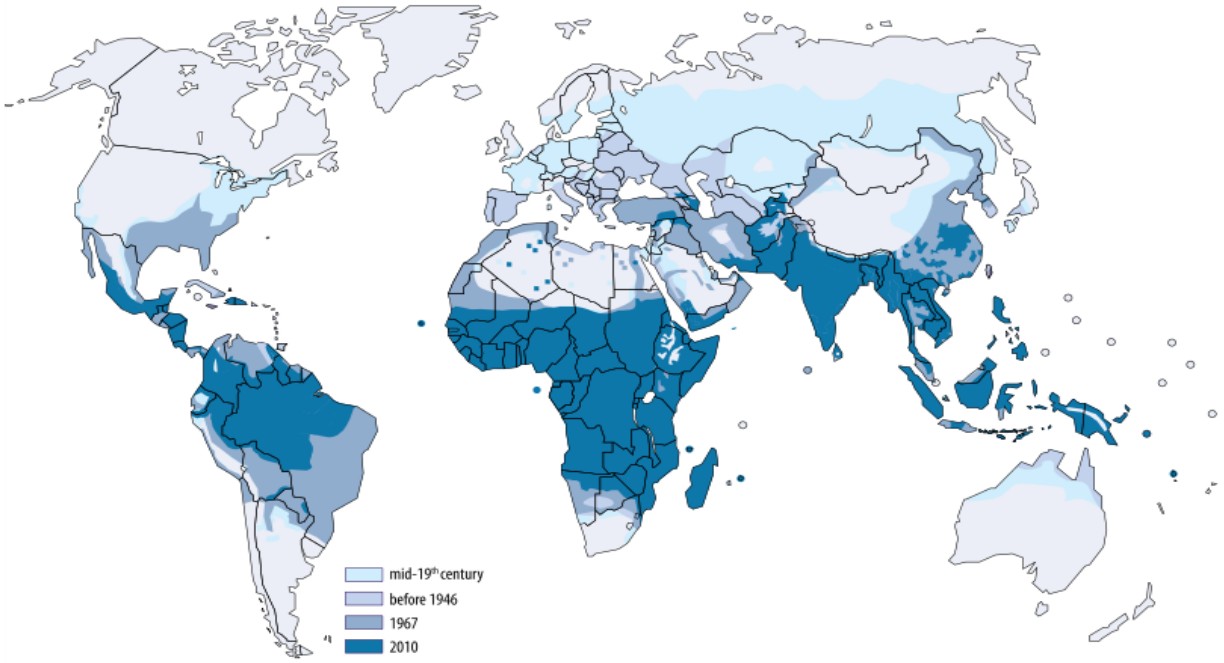

Figure 3- Evolution of malaria risk areas from the mid – 19th century until 2010.
Source: (Mendis et al., 2009; World Health Organization, 2011).

Box 2 | **Indoor Residual Spraying**

Dichlorodiphenyltrichloroethane (DDT) is a molecule known for its insecticidal properties. First synthesized in 1874, its insecticidal action was discovered by the Swiss chemist Paul Hermann Müller in 1939, for which he was later rewarded with the Nobel Prize of Medicine. DDT was then used in the second half of World War II to control malaria and typhus among civilians and troops.
DDT turned out to be such a potent insecticide that after the war it was made available for agriculture use, to control plagues at large scale, which led to the emergence of resistant mosquitoes. In the 1960's the public awareness for the very negative issues of the use of DDT led to the prohibition of its indiscriminate use. Before DDT, in tropical areas of Brazil and Egypt, malaria was controlled by poisoning the natural reproductive habitats of mosquitoes by applying, for instance, Paris Green in stagnant water, which was very toxic. Nowadays other classes of molecules exist such as carbamates and pyrethroids, but DDT remains on of the cheapest insecticide.

Several initiatives were developed in order to stop the transmission of the disease. The Millenium Developmental Goals form the United Nations (http://www.undp.org/) and is the Malaria Atlas Project, which analysis and maps the spatial distribution of malaria and the climate changes to predict malaria distribution (Figure 4) (Guerra et al., 2007)

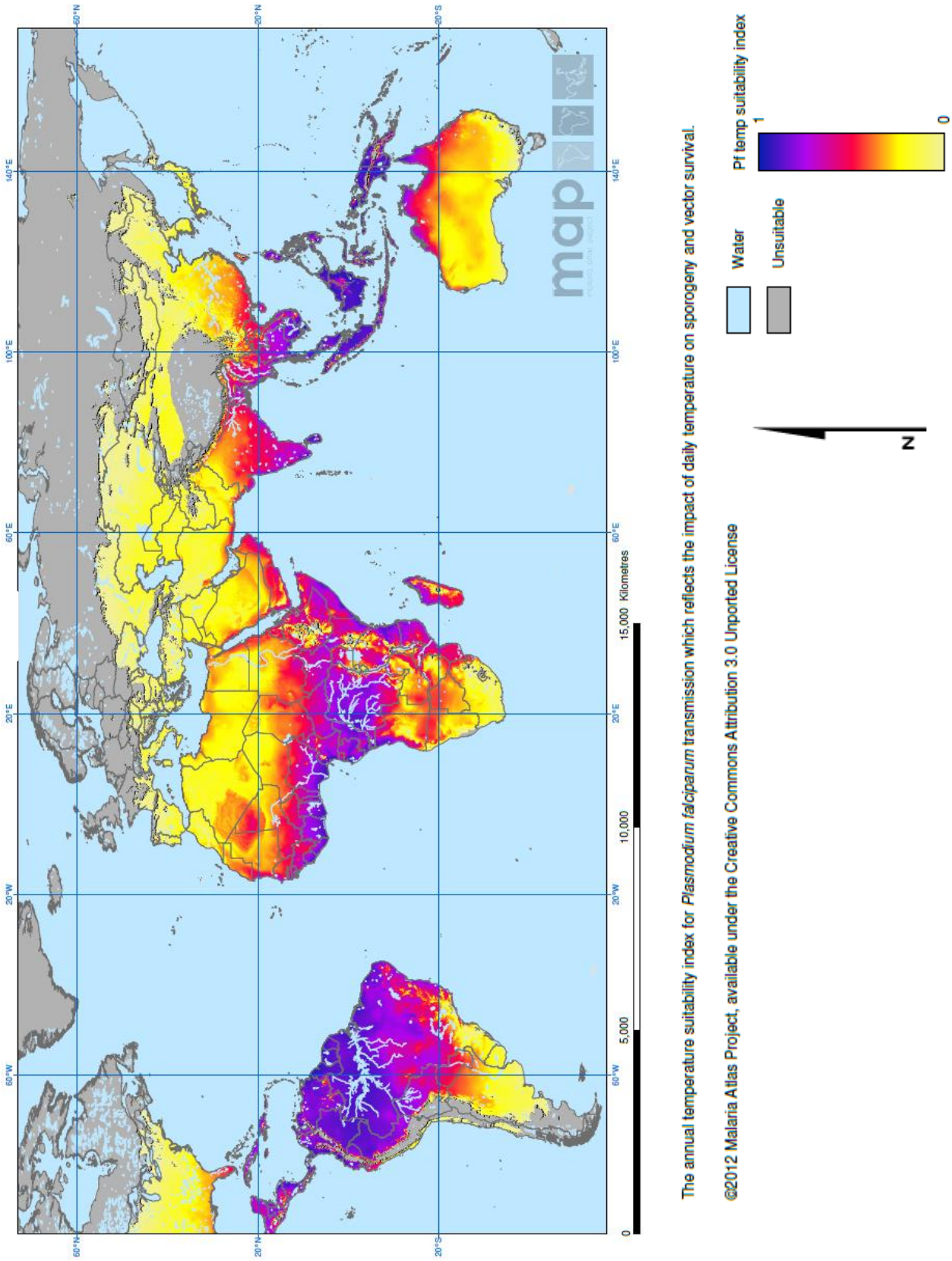

Figure 4 - Prediction of the transmission of P. falciparum according to the climate temperature.

Source: Malaria Atlas Project (MAP) http://www.map.ox.ac.uk/ (Gething et al., 2011b)

Worldwide researchers have focused their energies in several directions to cover the largest number of possible strategies to discover novel ways to cure/eradicate malaria. These strategies are vaccination development and the discovery of new drugs and new drug targets that haven't yet suffered from drug pressure. In the fight against malaria, all eyes are turned towards the commercialization of a vaccine that would interrupt the transmission of the disease (World Health Organization, 2013b).

I.3.4- The vaccine

Vaccines are the most cost-effective way to control and prevent a disease. So far a vaccine has never been produced against a parasite. It is particularly difficult to produce a vaccine against malaria due to: i) the variable surface antigens; ii) the genetic variability and the complex life cycle (multistage and multiantigene); iii) the high replication rate; and the rapid evolution of an immune evasive strategy by the parasite (Takala and Plowe, 2009; Takala et al., 2009; Weedall and Conway, 2010). To evade the immune system the parasite has developed astonishing strategies: i) all of the life cycle stages in humans are haploid for rapid selection and transmission of an advantageous mutation; ii) all replication is done intracellularly, to avoid contact with the immune system in this delicate phase and to disseminate rapidly (Mackinnon and Marsh, 2010); iii) merozoites infect red blood cells in less than 30 seconds, leaving little time for the immune system to react (Gilson and Crabb, 2009); iv) and the human immune system imposes a selective pressure that favors the emergence of extensive polymorphism and new antigens (Epstein et al., 2007; Mackinnon and Marsh, 2010). Highly conserved regions have probably not been under this selective pressure, and therefore will be good candidates to the development of a vaccine (Riley and Stewart, 2013).

In the first half of the 20th century, the first attempt to trigger immunization was done by the injection of inactivated sporozoites, the Pasteur vaccine approach (Good, 2013). Unfortunately this attempt was not successful. 30 years later, this approach was revisited: the sporozoites were directly dissected from the mosquito salivary glands, irradiated and cryo-preserved before a subcutaneous injection. This efficient challenge technique in triggering a malaria immune protection by imitating, in a controlled way, the natural route of a human infection (mosquito sporozoite subcutaneous injection) (Epstein et al., 2011; Luke and Hoffman, 2003; Roestenberg et al., 2013; Seder et al., 2013; Vanderberg, 2009). Though, this technique was successful in immunizing people, it was not realistic for a vaccine elaboration. Improvements can be explored because a whole-parasite vaccine has theoretically its advantages, as "all" the parasite proteins are present, reducing the effect of polymorphism in a protein (Good, 2013). Between 1917 and the 1940's, *P. vivax* was used as an immune-therapy. The deliberate injection of parasites in healthy volunteers was done in order to obtain an immunization and elaborate a vaccine. This technique was invented by Julius Wagner von Jauregg, for which he received the Nobel Price of Medicine in 1927. However, this technique was very dangerous, killing 15% of healthy people (Vogel and Roberts, 2011).

In the 40's a success in preventing malaria in primates was achieved by injecting inactivated sporozoites combined with an immune-stimulating adjuvant. Unfortunately this adjuvant was toxic and the sporozoites were obtained from human blood, making this approach impracticable (Clyde et al., 1973a, 1973b; Freund and Thomson, 1948; Nussenzweig et al., 1967; Rieckmann et al., 1974).

With the advances of the molecular biology techniques, it was possible to produce recombinant parasites proteins and culture techniques were considerably improved.

There is actually no vaccine commercially available, although almost 20 candidates are being tested in clinical trials (see example in Table 2). Optimism is justified as an acquired immunity against the disease is observed in endemic countries in healthy carriers. Children that survived an episode of severe malaria acquire protective immunoglobulins (IgG) and rarely develop another episode. Immunity against mild malaria is also observed (Cohen et al., 1961). Researches show that if some immunoglobulin is taken from these people and injected into individuals that have no protective immunity, some immunity is acquired (Cohen et al., 1961; Sabchareon et al., 1991).

I.3.4.1 - Potential targets

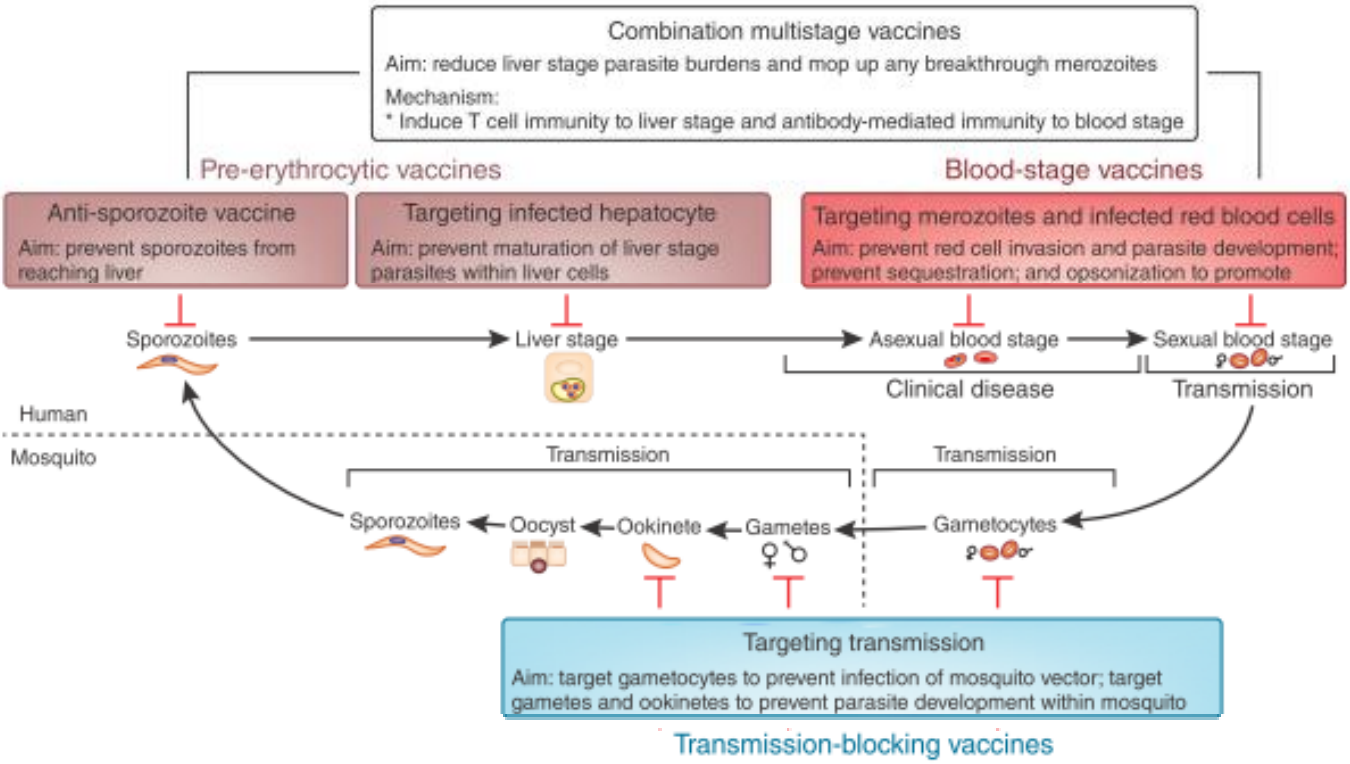

Figure 5 - Malaria vaccine approaches and targets.

Adapted from (Riley and Stewart, 2013). *TEM- effector memory T cells, TCM – central memory T cells.*

The complex life cycle of *Plasmodium* has been one of the major difficulties encountered in the development of a vaccine. But it is also this complex life cycle that gives so many possible targets and stages to explore (Figure 5 and Table 1).

1. Targeting the very first steps of infection from sporozoite inoculation after mosquito bite to the hepatocyte invasion, or even the infected hepatocyte itself, would confer a "sterile immunity", *i.e.* transmission blocking (Duffy et al., 2012; Heppner, 2013).

2. Developing a vaccine against blood-stages like the: erythrocyte invasion, merozoite replication, adherence of erythrocytes to blood vessels walls, or even against infected erythrocytes; could prevent the development of the disease. This would help protecting people from developing symptoms of the disease. Although a rapid emergence of escaping haplotypes can arise from this vaccine (Riley and Stewart, 2013).

3. Designing a vaccine against gametocytes could prevent new infections and hence stop the spreading of malaria. Though this option would not confer any protection to the individual itself, but it will help controlling malaria at a population level. This could be achieved by targeting directly the gametocyte, blocking fertilization or blocking the development in the mosquito mid-gut. The advantage is that gametocytes are poorly polymorphic.

It is important to understand the essential immune effectors, triggering an immune response in humans, to find new antigens that could be exploited as vaccines (Riley and Stewart, 2013).

Targets	Mechanisms of action	Reference
Protein kinases	Are present all through the parasite life cycle	(Zhang et al., 2012)
P. falciparum reticulocyte-binding protein homologue 5 (PfRH5)	An essential protein for erythrocyte invasion, with little genetic diversity	(Crosnier et al., 2011; Douglas et al., 2011)
Duffy Binding Protein (DBP) of *P. vivax*	Binds to the Duffy Antigen (DARC) of erythrocytes during invasion of the red blood cells.	(Batchelor et al., 2014)
Variant surface antigens of *P. falciparum* PfEMP with a conserved region of the highly polymorphic VAR2CSA gene	Responsible for adhesion of the infected red blood cells to the placental sulfate A. One was found to be a key target of the immune response, and antibodies against PfEMP1 can confer immunity in children. It is a good candidate to prevent placental malaria	(Chan et al., 2012; Fried and Duffy, 1996; Fried et al., 2013; Salanti et al., 2003)

Table 1 - Some targets exploited for vaccine development.

Perhaps different types of vaccines could be developed, depending on the target population and the region of the globe. We can imagine a vaccine for the populations mostly exposed to *P. falciparum,* or *P. vivax;* or a vaccine for travelers to immunize people that were never in contact with the parasite reducing a related public health problem, of cost and side effects of malaria prophylaxis; or developing a tissue-specific vaccine; or adding several antigens in the same vaccine. However these are utopian considerations as no vaccine is yet available.

Some vaccines are under development and actually in clinical trials. In the table below we present some examples of these developed vaccines (Table 2).

Vaccine	Composition	Clinical Trial Phase	Reference
SPf66	One of the earliest vaccines developed. It is a synthetic peptide vaccine containing antigens from blood stages linked together with an antigen from sporozoite stages	Extensively tested in clinical trials in endemic regions but inefficient	(D'Alessandro et al., 1995; Graves and Gelband, 2006)
PfCSP	Targets recombinant Circum-Sporozoite Protein (PfCSP)	Did not show any protective immunization during clinical trials, but is being improved	(Hoffman et al., 1994; Le et al., n.d.; McCoy et al., 2013)
NYVAC-Pf7	Targets multi-stages combining 7 *P. falciparum* antigens, from various stages of the life cycle (sporozoite, liver, erythrocytic and sexual stages).	Cellular immune response of over 90%, in contrast the antibody response was very low. This vaccine is worth more tests.	(Stoute and Ballou, 1998; Tine et al., 1996)
[NANP]19-5.1ANP	Schizont export protein 5.1 and 19 repeats of the sporozoite surface protein	Successful in conferring immunity in children and needs more clinical trials	(Reber-Liske et al., 1995)
Pfs25 and Pvs25	Malaria transmission blocking vaccine. Antigens conserved between *Plasmodium* species, interfering with oocytes development in the mosquito mid-gut	Phase 1 of clinical trials.	Dinglasan et al 2008 www.malariavaccine.org
AMA-1/ AS02	Targets blood stages, confers safe and immune protection in adults, however highly polymorphic and would need to be formulated in a multiallele vaccine	Phase 2	(Ouattara et al., 2013; Thera et al., 2011)
attenuated sporozoite vaccine	Developed for the first time in the 1940's. The procedure was ameliorated by establishing a manufacture process to aseptically irradiate and cryopreserve sporozoites.	Under phase 2 clinical trials (NCT01441167). The intravenous route was found more efficient in triggering an immune response. Practical issues are still pending for delivering intravenously this vaccine on the field, and overcoming the maintenance of a cold chain with liquid nitrogen	Sanaria Inc, (Alonso and Tanner, 2013; Epstein and Richie, 2013; Epstein et al., 2011, 2007)
VMP001	*P. vivax* vaccine. chimeric CSP protein with repeated regions of two major alleles VK210 and VK247, with AS01 adjuvant	Phase 2 of clinical trials	(Yadava et al., 2007)
RTS,S/AS01	Pre-erythrocytic vaccine constituted of a portion of the circumsporozoite protein (PfCSP), the major coat protein of the invasive sporozoite, which contains B-cell epitopes, and the C-Terminal T-Cell epitopes (T), fused to the Hepatitis B surface antigen (S), co-purified with additional "S" particles, coupled to an adjuvant AS01. The CSP antigen is capable of triggering the production of antibodies preventing the invasion of hepatocytes and destroying infected hepatocytes.	Phase 3	(Alloueche et al., 2003; Bojang et al., 2001; Kester et al., 2009; Regules et al., 2011; Stoute and Ballou, 1998; Stoute et al., 1997)

Table 2 - Some vaccine candidates under development.

The most recent hybrid subunit recombinant vaccine RTS,S/AS01 is the most advanced candidate that completed phase III of clinical trials in 2011. This vaccine should be available soon and would be used in addition to traditional preventive, diagnostic and treatment measures. The estimated efficacy for children 5 – 17 months age is of 55% reduction of the malaria episodes during the first 12 months, and 47% efficacy against severe malaria. 18 months after immunization the efficacy drops to 46% and 35.5%, respectively. For children aged between 6 – 14 weeks the vaccine is less efficient, reducing cases by only 25% (Agnandji et al., 2012, 2011; Regules et al., 2011; Riley and Stewart, 2013; World Health Organization, 2013c). Although the efficacy and the duration of protection are limited,

it is the best candidate nowadays. The RTS,S/AS01 (commercial name: Mosquirix) will be available for African countries with no profit as it is part of the malaria eradication project. It will be available in 2015, regarding approval from the African governments.

Malaria Vaccine Technology Roadmap (funded by Bill and Melinda Gates Foundation) set two major goals for a vaccine with a minimum 50% of efficacy against severe malaria in 2015. A second generation candidate with 80% of prevention of clinical malaria episodes would be needed by 2025 (Heppner, 2013; Heppner et al., 2005; Riley and Stewart, 2013; Vannice et al., 2012) www.malariavaccine.org).

I.3.5 - Antimalarial drugs

The first efficient treatment against malaria was made from the bark of Cinchona tree. This tree grows in the slopes of the Andes, especially in Peru. Indians used it to make a tincture to treat fever. In 1640 it was discovered to be efficient against malaria and was introduced by Jesuits in Europe. Only in 1820 the active ingredient was extracted and isolated from the Cinchona's bark and identified to be quinine, by two French chemists: Pierre Joseph Pelletier and Joseph Bienaimé Caventou (Kyle and Shampe, 1974).

Quinine became the principal antimalarial treatment until the 1920's decade. In the 1940's, quinine is substituted by chloroquine. This new treatment was largely administrated in Southeast Asia and South-America in the 1950's, and expanded to the entire world in the 1980's (Achan et al., 2011). Then in the 1970's artemisinin molecule was discovered from the plant *Artemisia annua*, and became the most widely administrated drug for malaria treatment (Tu, 2011) (see below section I.5 – Artemisinins, for more information).

Antimalarials used at the present time can be divided into three major classes: Quinolines and derivatives (Hemozoin inhibitors), Antifolates combination drugs, Artemisinin and derivatives (Sesquiterpene lactones) (Grimberg and Mehlotra, 2011) (Table 3, Figure 6). Few of these drugs possess an identified target.

Individual drugs	Hemozoin inhibitors	Aminoquinolines	Amodiaquine, Chloroquine, Primaquine, Pamaquine
		4-methanolquinolines	Mefloquine, Quinine, Quinidine
		Others	Lumefantrine (combined with Artemether), Halofantrine
	Antifolates	DHFR inhibitors	Pyrimethamine, Proguanil, Chlorproguanil, Biguanides
		Sulfonamides	Sulfadoxine, Sulfamethoxypyrazine
		Co-formulation	Sulfadoxine/Pyrimethamine (SP)
	Sesquiterpene lactones		Artemether, Artesunate, Dihydroartemisinin, Artemotil, Artemisinin
	Others		Atovaquone (with Proguanil as Malarone®), Tetracycline, Doxycycline, Clindamycin, Pyronaridine, Piperaquine
Combination therapies	Fixed-dose (co-formulated)		Artemether/Lumefantrine - Artesunate/Amodiaquine (ASAQ) Dihydroartemisinin/Piperaquine - Artesunate/Pyronaridine Artesunate/Mefloquine(ASMQ)
	Others		Artesunate/SP - Artesunate/Mefloquine - Quinine/Tetracycline Quinine/Doxycycline - Quinine/Clindamycin

Table 3 – Main classes of antimalarials.

Source: (Grimberg and Mehlotra, 2011).

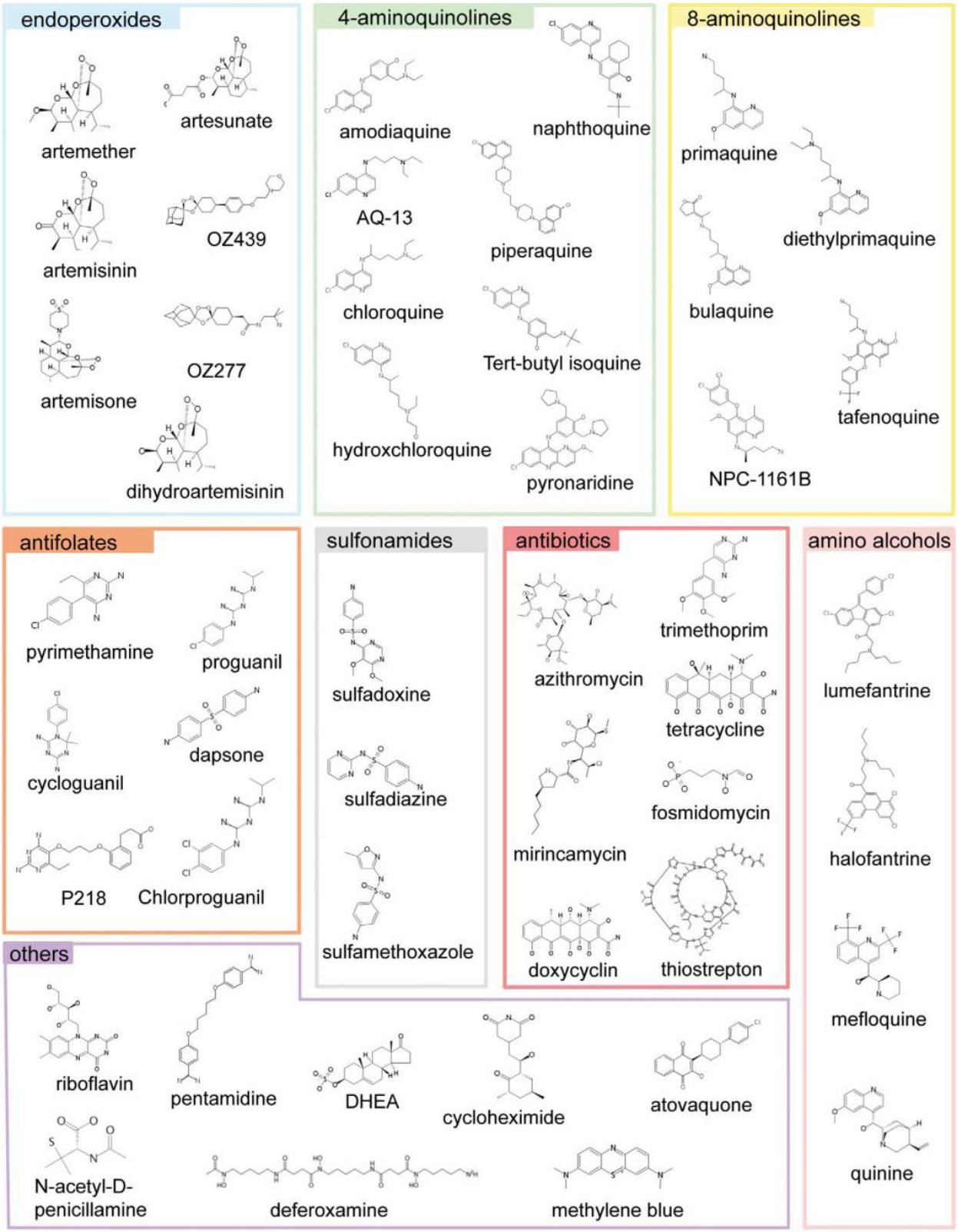

Figure 6 - Structures of some antimalarials, organized by major chemical family.

Some chemical structures of the main classes of antimalarials and other therapeutic and control molecules, are assembled according to either their chemical classes (endoperoxides, 4- and 8- AQs, amino-alcohols), their function (antifolate, antibiotics), or both (e.g., sulfonamides, a chemical class of antibiotic used in combined antimalarial therapies). Source: (Delves et al., 2012).

Chloroquine (CQ) was the safest, cheapest and it is one of the most efficient antimalarial drugs until resistances appeared. However, it is still widely used, especially in combination with other drugs. Antifolate Sulfadoxine/Pyrimethamine (SP) combination drug is one of the other widely used inexpensive antimalarials (Fidock et al., 2004). Amodiaquine and Mefloquine as well as chloroproguanildapsone (LapDap, another antifolate drug) were used after CQ and SP resistances appeared (Fidock et al., 2004). Mefloquine was used during the Vietnam War to treat American troops against multi-resistant malaria. Nowadays it is also used in prophylaxis. Atovaquone is used in combination with Proguanil for travelers malaria prophylaxis, also known under the commercial name of Malarone®. Primaquine is used for the treatment of malaria relapses in *P. vivax* infections. Antifolate drugs are used in combination with dihydrofolate reductase inhibitors (DHFR) and dihydropteroate synthase inhibitors (DHPS). Artemisinin was first a natural drug isolated from the Chinese herb Qinghaosu - *Artemisia annua* (Grimberg and Mehlotra, 2011). Proguanil/cycloguanil and pyrimethamine (Peterson et al., 1990), sulfadoxine (Wang et al., 1997), and atovaquone (Srivastava and Vaidya, 1999), respectively target dihydrofolate reductase (DHFR), dihydrofolate synthetase (DHPS) and cytochrome b (McNamara and Winzeler, 2011).

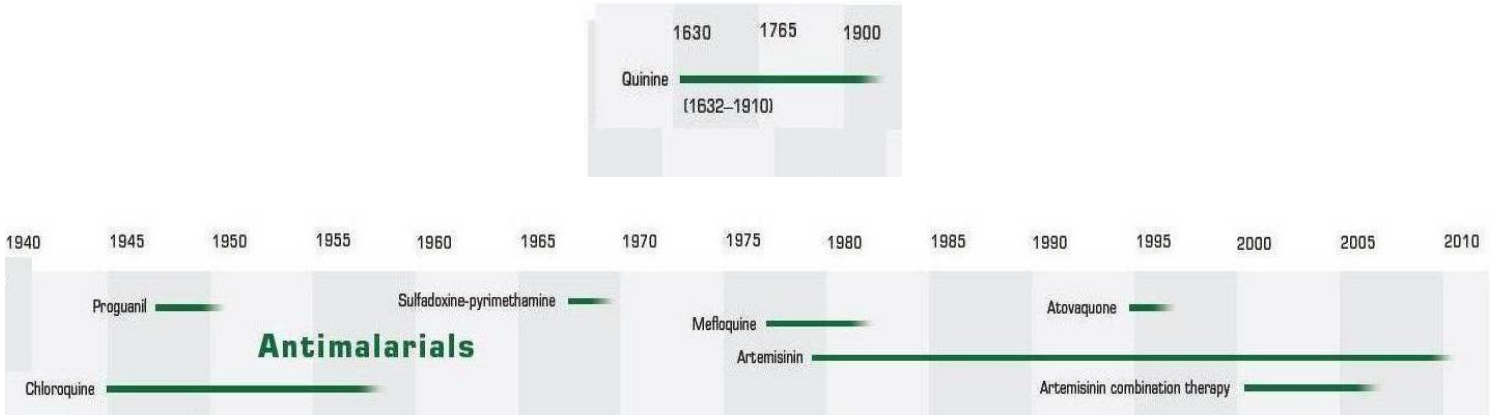

Figure 7 - Timeline of the discovery of new antimalarials and the emergence of the first resistances.

Source: Annex B of "The Race Against Drug Resistance". Mead Over http://www.cgdev.org/blog/antiretroviral-drugs-will-retain-their-power-longer-if-donors-and-gove.

I.3.7 - Antimalarial resistance

Some of these drugs have been used for decades and unfortunately parasites have developed resistance mechanisms against some quinolines and antifolates (Grellier et al., 2012; Grimberg and Mehlotra, 2011) (Figure 7). After the increasing death of children under 5 years in sub-Saharan African countries due to chloroquine and sulphadoxine-pyrimethamine resistances emergence, WHO endorsed a policy for using artemisinin-combination therapies (ACTs) as first line treatments, decreasing a lot mortality, along with the introduction of other prevention measures (Dondorp et al., 2010; Korenromp et al., 2003). However this tendency is starting to be reverted with the rising of artemisinin resistant strains in Thai-Cambodian border since 2004 (Alker et al., 2007; Denis et al., 2006; Dondorp et al., 2010; World Health Organization, 2007).It is now more than ever urgent to discover affordable alternatives to the available treatments.

It has been suggested that parasites genetic backgrounds can influence the response to certain antimalarials and the effect of: *pfmdr1* (Hunt et al., 2007; Reed et al., 2000; Sidhu et al., 2005) and *pfcrt* (Stephanie G Valderramos et al., 2010) on CQ resistance; *pfnhe1* on quinine resistance (Briolant et al., 2011; Cui et al., 2012; Meng et al., 2010).

Resistances in the malaria parasite are particularly prone to emerge due to its genetic and genomic plasticity. It is then important to stay one step ahead of the parasite, in terms of new antimalarials or predicting target mutations to be able to react when such occurs. Though it is not easy and is a very long process.

1.3.8 - Finding antimalarials mode of action

Finding antimalarials mode of action is a rather difficult task and it can be multarget. These can be achieved by several ways:

- *In vitro* induced drug resistance and/or using field resistant isolated parasites, and by Genome Wide Screening (GWS) try to find mutations only present in resistant parasites. If this is achieved one can learn more about resistant mechanisms or the direct target of the drug (Ariey et al., 2014; Rottmann et al., 2010); another possibility is to demonstrate resistance on a transgenic parasite where the candidate target has been mutated (e.g. the mutated dihydrofolate reductase gene conferred resistance to pyrimethamine (Wu et al., 1996).

- Others focus on trying to find the specific stage of the parasite cycle in which the molecule acts (Delves et al., 2012) (Figure 8), and then try to infer the target by stage specific gene expression profiles.

- Another way is by finding specific phenotypes or mechanisms disruption, induced in parasites after drug treatment. One way to test if a molecule with antimalarial activity is targeting a specific pathway or protein, and by repeatedly showing that this molecule has the same effect as a known inhibitor of the target. One can also find a phenotype observable when a

protein or a pathway of the parasite is inhibited (swollen food vacuoles of parasites treated with cystein proteases (Rosenthal et al., 2002).

After the target is identified it can be heterologously expressed and purified; a model for a targeted pathway can also be elaborated; *P. falciparum* genes encoding for drug targets can be expressed in *P. berghei,* for which the transfection technology has been developed, and as *P. berghei* infects mice, an *in vivo* model is then available.

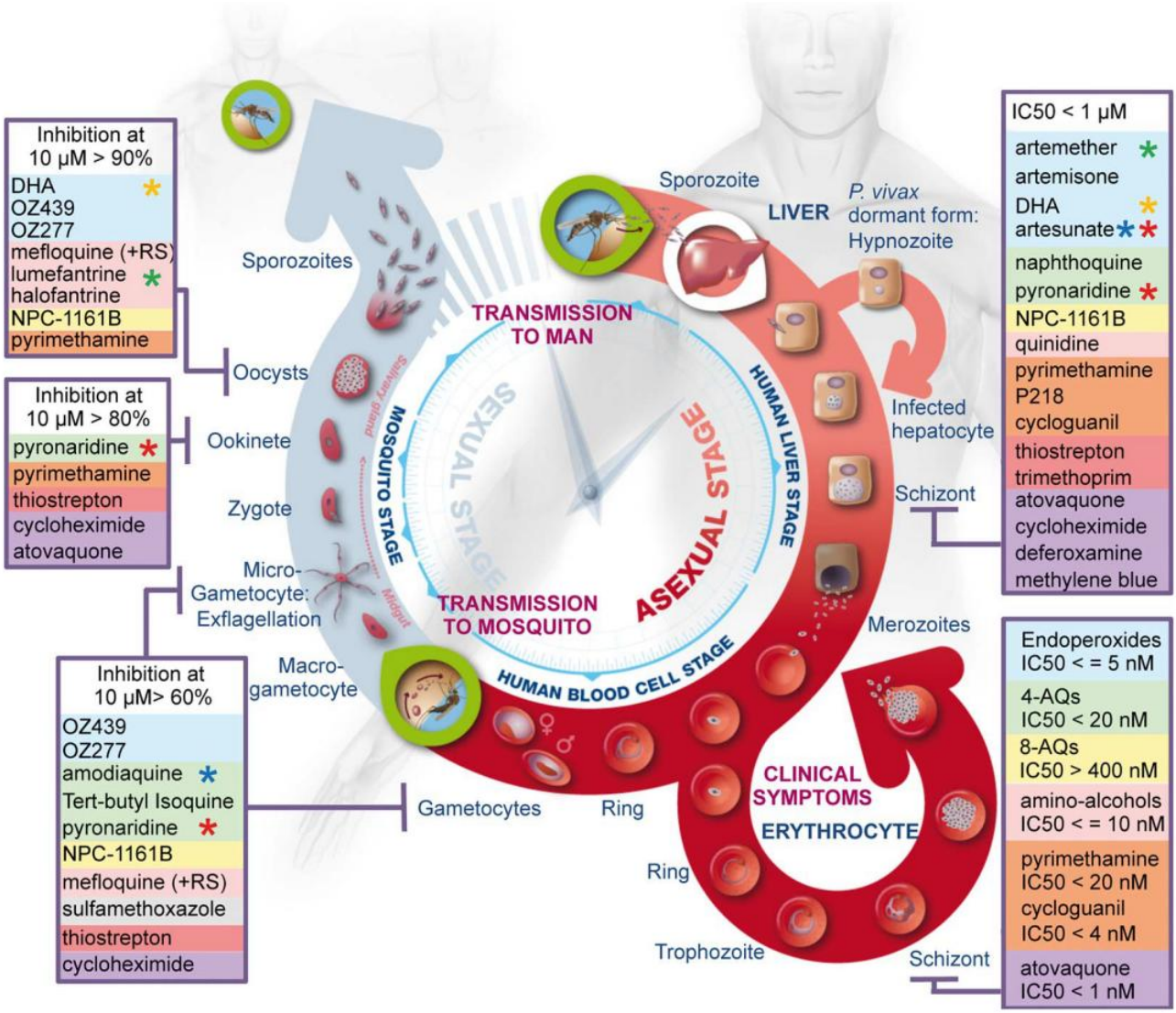

Figure 8 - Summary of stage-specific activity of the most common antimalarials.

The three major phases, i.e., liver stage, blood stage, and vector stage, of the life cycle of Plasmodium are shown. The two key entry points leading to transmission of the parasites from vector to host and from host to vector are indicated by the green circles. For each developmental stage, the identified actuating drugs are listed in boxes and coloured as described in Figure 6. Stars indicate components of the main artemisinin combination therapies used: green - coartem; red -pyramax; orange - eurartesim; blue - ASAQ. DHA – dihydroartemisinin. Source: (Delves et al., 2012).

I.4 - New Antimalarial Research

I.4.1 – Strategies for new antimalarial discovery

Malaria has threatened mankind for thousands of years. Even though the prevention initiatives have diminished a lot the number of malaria cases, it is still essential to develop treatments for infected people (especially children and expectant women) and, with the emergence of artemisinin resistance, new antimalarials are urgently needed (Wells and Poll, 2010). Most of the potent antimalarials that we nowadays use were not elaborated with a specific drug target but instead were found from natural products that presented an antimalarial activity (quinine and artemisinin), or semi-synthetic derivatives (chloroquine and artesunate), or even active against other pathogens (antifolates and tetracycline).

Several tools exist that can be exploited for new antimalarials research. Sequencing of *P. falciparum* genome revolutionized the study of malaria and drug discovery (Gardner et al., 2002) (http://plasmodb.org/) and new tools exist nowadays for the annotation of unknown genes, such as biological data, knowledge charing from the scientific community and the 2007 PlasmoExplore consortium (Florent et al., 2010). Databases have been created such as TDR targets (http://tdrtargets.org) that can be used to predict new targets *in silico;* or the *Plasmodium* Protein Data Bank (www.pdb.org) that gathers information about proteins and their structures.

Mainly two global approaches have been defined for antimalarial research:

- The screening of molecules issued from chemical libraries either on a phenotypic way, *i.e.* testing compounds upon *in vitro* cultures of *Plasmodium* (whole cell approach) (Table 4);
- Searching for inhibitory molecules that act upon the biochemical activity of a potentially identified drug target that can be an essential enzyme or pathway, ideally specific to the parasite (target based approach) (Chatterjee and Yeung, 2012) (Table 4);

	Target-based approach	Cell-based approach *(whole cell)*
Characteristics	• Identification of a target from bibliography, orthology with targets form other organisms, genomic data • Genetic and chemical validation of the target • Target expression and eventually purification • Development of a high-throughput compound screening test on the target • Compound testing *in vitro* on *Plasmodium,* cytotoxicity tests on mammalian cells, *in vivo* testing • Compound optimization	• Identification of libraries of potential antimalarial molecules • High-throughput compound screening test *in vitro* on *Plasmodium,* cytotoxicity tests on mammalian cells, and *in vivo* testing • Compound optimization
Pros	Known mechanism of action, possibility of optimization with target knowledge	Active on *Plasmodium* (cell penetration), the target can be later identified by several tools
Cons	Difficulties in expression and eventual purification of the target; genetic and chemical target validation required, development of a specific high-throughtput screening test required	Mechanism of action unknown, optimization can be difficult

Table 4 - Approaches for antimalarial discovery.
Adapted from: (Chatterjee and Yeung, 2012).

But other strategies to find new antimalarials exist such as: (Grellier et al., 2012)

- New drug design from the knowledge of a target;
- The optimization of already existing antimalarials by drug combination, rearrangements/modification of existing compounds in order to limit the side effects of these;
- Multi-target hybrid antimalarial design or new combinations of already existing antimalarials (polypharmacology), overcoming antimalarial resistance issues when only one target is aimed (Morphy and Rankovic, 2005);
- Adaptation of drugs used to cure other pathogens or diseases such as cancer; exploring natural products;
- Or even target validation upon orthologs of other Apicomplexan parasites that present evolutionary relation and are technically easier to manipulate, like for example *Toxoplasma gondii*.

Although it is not required to know the target of an antimalarial, it is rather useful because if this molecule fails clinical trials it is always possible to explore this target in future compound research, and also to predict potential resistances (McNamara and Winzeler, 2011).

Going backwards and identifying the unknown targets of established antimalarials can also be useful. These can be then exploited to design and test new molecules with more affinity to the target, or by targeting a different binding site of the same validated target, and hence exploring a region that has not suffered from drug pressure. For example, CQ, that was discovered to interfere with hemoglobin degradation pathway, this is now a validated target explored for other antimalarials (Biagini et al., 2003; Ursos and Roepe, 2002). Structural knowledge of the target will help to design new antimalarials, exploiting the regions non- homologous to host proteins.

Common sense tells us that a future good target should be a feature absent in humans (e.g.: the prokaryotic apicoplaste), or an ortholog with very different characteristic. In order to avoid toxicity and to be able to specifically target the *Plasmodium* feature. The only difficulty is validating the target from no previous information. If one can exploit the percentage of difference between these two orthologues and extract molecules acting specifically on *Plasmodium,* then there is no danger in using orthologues proteins. There are even advantages in this procedure that rely on the eventual knowledge of the human orthologue. A validated target in another organism (human or other pathogen) than *Plasmodium* can also be exploited (Fidock et al., 2004). Some examples of this strategy is the targeting of cysteine proteases by a cysteine protease cathepsin K, which is a treatment against osteoporosis (Rosenthal et al., 2002; Rotella, 2002), or targeting the farnestyl transferases inhibited by molecules against the human orthologue used in cancer treatment (Figure 9) (Chakrabarti et al., 2002; Gelb and Hol, 2002).

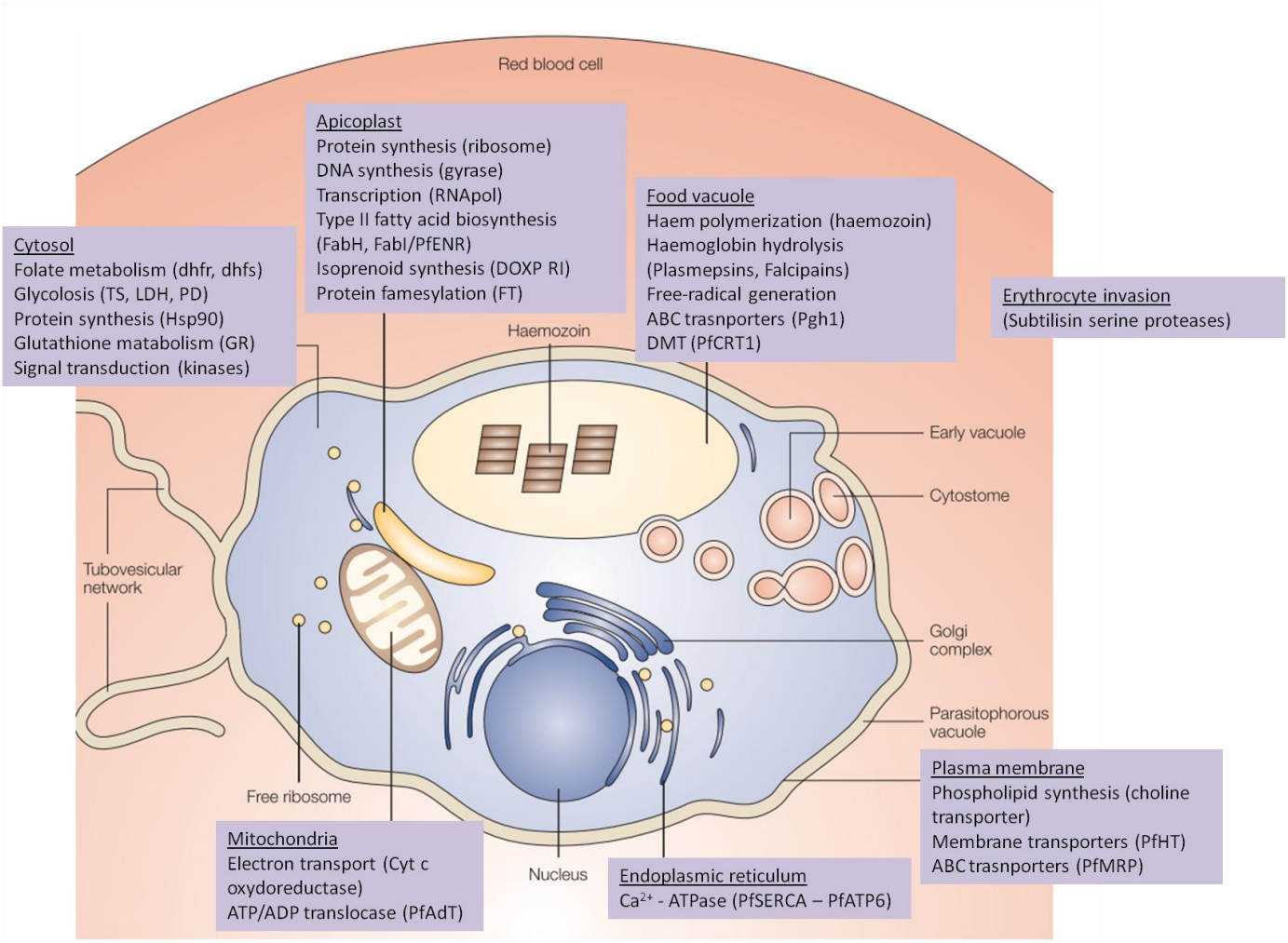

Figure 9 - Some targets explored for new antimalarial research.

Adapted from (Fidock et al., 2004).

I.4.2- New targets explored

Some examples of the targets explored are cited below. Drug discovery strategies that are focused upon exploring new targets have a vast choice of targets that can go from physiological/metabolic pathways to the study of single proteins such as transporters and kinases and pathways such as the haeme polymerization mechanism (Grellier et al., 2012; Grimberg and Mehlotra, 2011).

i) Transporters are integral membrane proteins and enable the transport of solutes across biological membranes. They are promising targets as they are generally involved in essential pathways and resistant mechanisms, such as PfCRT, Pgh1 and PfNHE1. Some transporters have been localized and some have been heterologously expressed as recombinant proteins.

ii) Parasites lipidome and lipid synthesis (Maréchal et al., 2011) are good drug targets because, post invasion of the host cell, innumerous membranes are synthethized during rapid cellular

division, maturation, and creation of membranous networks between the host cell and the parasite in *Plasmodium*

iii) Kinases are involved in host-parasite interactions and the *Plasmodium* kinome (85 – 99 enzymes) is different from the human kinome. Several families can be exploited: calcium-dependent kinases (CDPK), Apicomplexan specific kinases family (FIKK), that are absent in humans.

iv) Proteases are involved in innumerous pathways and hence could be good potential drug targets. During the erythrocytic stages of *P. falciparum* development, hemoglobin is degradated by proteases as a major source of amino acids for parasite development. This process occurs in the acidic food vacuole and generates free haeme that is toxic for the parasite. It is then polymerized into haemozoin, a non-toxic pigment. Avoiding hemoglobin degradation by cystein and aspartic antiproteases can be potential targets (Biagini et al., 2003; Deharo et al., 2002; Huy et al., 2007; Ncokazi and Egan, 2005; Rush et al., 2009; Wegscheid-Gerlach et al., 2010; Weissbuch and Leiserowitz, 2008). Inhibiting hemozoin formation pathway is a good target for antimalarial development. Several drugs already target this pathway such as chloroquine and amodiaquine. Other processes gene disruption, cystein proteases DPAP1 (kembla et al 2004), metalloaminopeptidases facilysin (Eggleson et al., 1999; Ponpuak et al., 2007) and PfA-M1 and PfA-M17 (Azimzadeh et al., 2010; Deprez-Poulain et al., 2012; McGowan et al., 2009; Skinner et al., 1996; Trenholme et al., 2010). PfA-M1 have been described as a promising antimalarial targets. PfA-M1 is a zinc-aminopeptidase and has been characterized during the erythrocytic cycle of *P. falciparum* (Azimzadeh et al., 2010; Florent et al., 1998) and chemically validated as a target with several promising inhibitors identified (Flipo et al., 2003; M Flipo et al., 2007; Marion Flipo et al., 2007).

v) The apicoplast is a vestigial, non-photosynthetic, plastid-like organelle inherited from the prokaryotic world by secondary endosymbiosis, and is essential and unique to Apicomplexan parasites. It has a 35-kb circular genome and several biochemical pathways that are absent in humans. Exploring these pathways as drug targets is very promising. Some examples are: HSP70 (70 kDa heat shock protein) essential for trafficking of proteins to apicoplast; pathways for fatty acid and non-mevalonate isoprenoid biosynthesis; partial haem synthesis enzymes (delta-aminolevulinate dehydratase ALAD); ferrodoxin-$NADP^+$ reductase and its redox partner; ferrodoxin organel specific biosynthesis pathway [Fe-S]; pyruvate dehydrogenase complex; and alfa-ketoglutarate dehydrogenase. Processes like apicoplast replication, prokaryotic protein synthesis (targeted by tetracyclines and clindamycin) or prokaryotic DNA and RNA machinery (targeted by quinolone and rifampicin) are also interesting targets (Grellier et al., 2012; Seeber and Soldati-Favre, 2010).

vi) Folate uptake is also essential for *Plasmodium* DNA synthesis and are already targeted by several antimalarials (antifolates such as Sulfadoxine/Pyrimethamine). Dihydrofolate reductase-thymidylate synthase (DHFR-TS) is a validated antimalarial target widely studied, and the structure has been solved by X-ray crystallography (see Staines et al 2010 for review).

I.4.3 - Biological target validation

A protein or a biological pathway has to be validated to be considered a drug target. It can either be by: i) genetic validation, by showing that a KO is deleterious for the parasite; ii) or chemically, with specific inhibitors for the target showing an inhibition deleterious for parasite survival (Staines et al., 2010). Both validations are complementary and not mutually exclusive.

Chemical validation has its limitations because it is difficult to prove that a molecule will only specifically inhibit this protein or pathway, by testing it on *Plasmodium* cultures or *in vivo* in a malarial model.

Genetic validation should then be achieved in parallel by deletion of the corresponding gene. If this protein or pathway is essential for parasite growth or survival then we have a good antimalarial target. Techniques to knock-out (KO) a gene and transfect rodent *Plasmodium* and *P. falciparum* have progressed a lot. It is possible to KO a gene at a chosen stage of parasite cycle (Lacroix et al., 2011); to conditionally eliminate a gene product by fusing a degradation domain and Shield ligand (Dvorin et al., 2010); or to act by compensation of a lethal phenotype that results from a KO of an essential gene, by expressing in trans a wild type version of this gene (Slavic et al., 2010); allelic exchange; or random mutagenesis by PiggyBac transposable element (Crabb et al., 2011; Grellier et al., 2012).

However, genetic validation is difficult because: i) RNAi cannot be achieved in *P. falciparum* since the enzyme required to degrade dsRNA is not present (Baum et al., 2009); ii) drug treatment does not induce significant changes in transcription (Ganesan et al., 2008; Kato et al., 2008; Tamez et al., 2008); iii) and nearly 50% of *Plasmodium* genes remain unannotated (McNamara and Winzeler, 2011).

Finally, *T. gondii* is a good model organism to study *Plasmodium* as it is an Apicomplexan, closely related to *Plasmodium* and has an extensive repertoire of experimental and genetic techniques (Kim and Weiss, 2008).

I.4.4- Compound screening

The erythrocytic stage is symptomatic and diagnosable. Many treatments are developed against this particular stage. Bioassays on *Plasmodium* erythrocytic stages are possible. A protocol for continuous culture of *P. falciparum* on human erythrocytes was established in 1976, and is nowadays a great tool for antimalarial research (Trager and Jensen, 2005). A protocol for continuous culture of *P. vivax* is not yet established, although short time enriched cultures of 4 weeks is possible (Udomsangpetch et al., 2008). *P. knowlesi* can be cultured on monkey erythrocytes (Kocken et al., 2002) but no continuous *in vitro* growth of *P. ovale* or *P. malariae* is nowadays possible (Grellier et al., 2012).

Drug or vaccine development against liver stages and gametocytes is also possible. Gametocytes can be obtained by stress conditions and maintained in culture, although a long maturation is required (>10 days) and alternative techniques are very expensive and require specific equipment. For liver stages, *in vivo* models are used such as *P. berghei* and *P. yoelii,* but also primary rodent hepatocytes

or hepatoma cell lines. Primary culture on human hepatocytes or cryopreserved sporozoites is opening new perspectives into liver stages research (Chattopadhyay et al., 2010).

Compound screening is achieved by the determination of the parasite growth in presence of several dilutions of a compound and dose response curves can be drawn to establish IC_{50} [4] values. This can be achieved following different protocols: i) measurement of the incorporation of radiolabeled nucleotide precursors; ii) colorimetric assays; iii) fluorometric and flow cytometry counting.

i) The method of Desjardins et al. has by principle the measurement of the quantity of a radiolabeled nucleotide precursor ([^{3}H]-hypoxanthine) incorporated by the parasite during its growth, in presence of serial dilutions of the molecule to test. Radioactivity counts directly reflect the growth of the parasite, since red blood cells have a reduced metabolism and do not possess a nucleus (Desjardins et al., 1979).

ii) Colorimetric assays are less expensive but also less sensitive than radioactivity. Giemsa stained parasites can be counted under a light-microscope but this can only be performed for small amount of molecules. The colorimetric detection of lactate dehydrogenase (LDH) is also used (Makler and Hinrichs, 1993; Makler et al., 1993). This test is based on the immunodetection of PfLDH with a monoclonal antibody (Druilhe et al., 2001; Grellier et al., 2012; Makler and Hinrichs, 1993; Makler et al., 1993). Nowadays there exist rapid immunoassays strips (OptiMal®). Another test that can be done is simply an ELISA that will quantify the *Plasmodium* histidine-rich protein II (HRP2). We can also find these tests in commercial kits (Noedl et al., 2002). However, they are now as sensitive as radioactivity, they remain less stable and not optimized for field utilization.

iii) Fluorimetric and flow cytometry techniques take advantage of the fact that erythrocytes are anucleated. DNA present from the parasite is labeled with fluorophores (e.g.: DAPI, Hoechst 33258). These tests are optimized for high throughput screening, and are sensitive and cost-effective (only a spectrofluorimeter and dyes are required) (Bacon et al., 2009; Baniecki et al., 2007; Bennett et al., 2007; Grellier et al., 2012). Flow cytometry is less frequently used as it is only suitable for moderate throughput assays, and cytometers are expensive machines. But this technique has its advantages as it allows access to much more information than just the parasite growth (Grimberg and Mehlotra, 2011).

The identified molecules to analyse are first tested on asynchronized *P. falciparum* cultures that contain all erythrocyte developmental stages, to determine IC_{50} values. To establish the stage specificity action of the best molecule hits, synchronized cultures are tested. Most experiments are undertaken for 24h – 72h (classically 48h) of parasite contact with the molecules. It is important to note that IC_{50} are subject to high variation within and between laboratories, due to the biological material, the culture conditions, the assay chosen, the manipulator and the laboratory material, so that absolute values are to be taken with caution.

[4] IC_{50} - half maximal inhibitory concentration – it is the required concentration at which a given tested compound inhibits 50% of a biochemical function or cell/parasite *in vitro* growth.

Molecules are also tested for their toxicity on mammalian cells (HeLa, Vero, fibroblast, hepatoma cells) before passing to *in vivo* testing. The viability and/or growth of these cells in presence of the compounds can be analyzed by several measurements such as: cell integrity (by tryptan blue or propidium iodide coloring); mitochondrial activity (colorimetric MTT assay), membrane integrity (by LDH passage), cell proliferation (WST assay kit), quantification of cellular proteins (sulforhodamine B assay), and ATP content (colorimetric/fluorimetric) (Fidock et al., 2004).

Then the molecules are tested *in vivo* for their effect to treat malaria in a rodent model (non-primate animal model, most frequently immunocompromized mice). *P. falciparum* does not infect mice, so other *Plasmodium* species have to be used: *P. berghei, P. yoelii, P. chabaudi* or *P. vinckei.* Results can be different from these *in vivo* assays when compared to *in vitro* values and also clinical trials performed on humans. The most commonly used protocol is on *P. berghei* with a four day administration by oral, subcutaneous or intravenous route. Parasitemia counts at the end of the treatment and 3-7 days after are compared to control, but also data from mice survival time and malaria relapse. These tests provide also information about bioavailabilty and potency of the molecule, and ED_{50} [5] and ED_{90} values can be calculated. *P. berghei, P. vinckei* and some strains of *P. yoelii* and *P. chabaudi* cause lethal infections*, P. yoelii, P. chabaudi and P. vinckei petterei* are cleared after an acute parasitemia (Grellier et al., 2012).

When an efficient and low cytoxicity compound is discovered and validated according to Medecine for Malaria Venture (MMV) requirements (Table *5*), these molecules can enter clinical trials phases (Figure 10).

New antimalarials	New combination drugs
• Efficient against drug-resistant *P. falciparum* parasites cure in reasonable time (≥ 3 days), • Low toxicity, • Suitable for small children and pregnant women, • Can be orally administrated • Affordable • Can be used as a preventive treatment (intermittent treatment[6]) • Can be rapidly available and suitable for emergency situations (single-dose treatment), • Appropriate packaging and formulation, • Can be used in prophylaxis for non-endemic travelers, • Effective against *P. vivax* and severe malaria	• Improved efficacy over isolated molecules, • Have at least an additive effect and maybe a synergistic activity, • At least one of the partners has to be efficient in resistance regions • Reduce the probability of rapid selection of resistant parasites, • Active against several developmental stages, reducing the quantities of each component used (lower toxicity and cost). • Ideally has a new feature and similar pharmacokinetics to avoid unequal drug pressure on the parasite.

Table 5 - Medicine for Malaria Venture (MMV) requirements for new antimalarials and combination drugs.
(Fidock et al., 2004)***.***

[5] ED_{50} / ED_{90} – effective dose, is the amount of a drug that produces respectively a 50% or 90% reduction of the disease in the population.
[6] Intermittent preventive treatment of malaria is a public health intervention recommended by WHO. The target publics are pregnant women,sewborn, infants and small children. It is a full therapeutic course of antimalarial prevention (normally sulfadoxine-pyrimethamine) in all areas with moderate to high malaria transmission in Africa. It is given at routine prenatal and post natal visits, regardless of whether the recipient is infected with malaria or not.

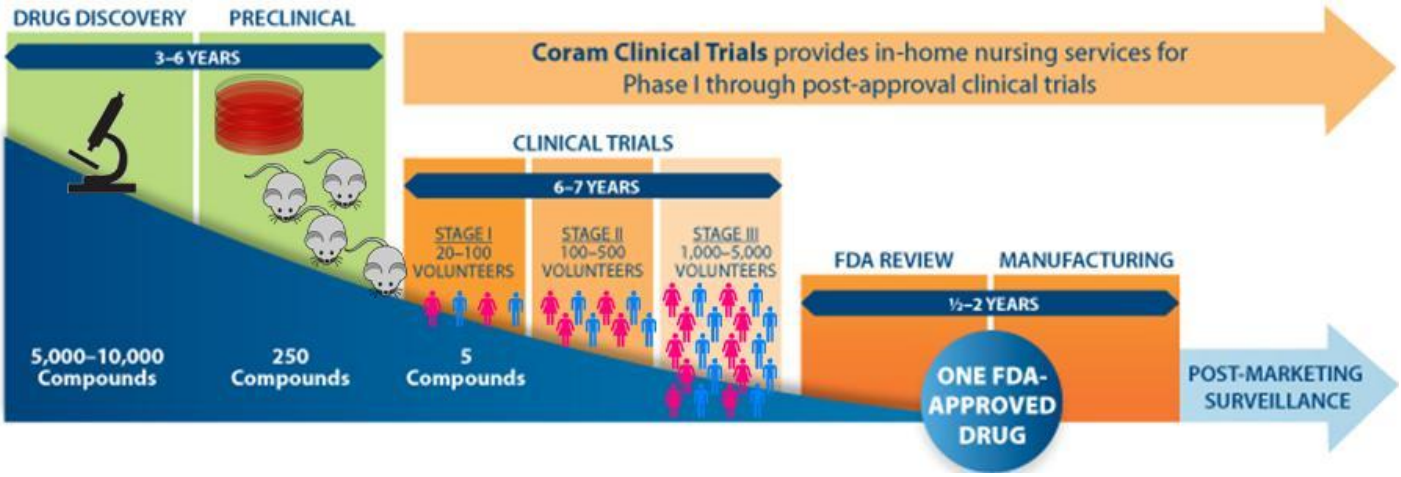

Figure 10 - Steps for new antimalarial discovery and development. *FDA – Food and Drug Administration*
Source: http://www.coramclinicaltrials.com/.

I.4.5 – New antimalarials discovered

Since 1996 no new antimalarials have been introduced in clinical practice. However, recently three novel drugs have been discovered and are now in Phase II of clinical trials (Charman et al., 2011). One of them, entirely synthetic peroxide, issued from a series of adamantine-based ozonides called trioxolane OZ439. The first molecules of this series (e.g. OZ277) had a short plasma half-life during clinical trials. Structural modifications led to OZ439 (Buckner et al., 2012). This molecule is active on parasites at a nanomolar range and has long half-life elimination. It is safe and well tolerated and can be administrated by a single oral dose (Charman et al., 2011; Mäser et al., 2012). The other molecule was identified after a whole cell approach screening from a library of natural products and synthetic compounds and is named NITD609, or spiroindolones. It is active at the nanomolar range, can be orally administrated, and presents neither cardiotoxicity nor genotoxicity. The P-Type sodium ATPase PfATP4 has been described as the target of spiroindolones based on drug resistance studies, although the exact mechanism of action remains unknown (Chatterjee and Yeung, 2012; Rottmann et al., 2010; Spillman et al., 2013; Mäser et al., 2012). AN3661 is another potent antimalarials developed by Ancor Pharmaceuticals that is active at the nanomolar range and is a boron-based drug candidate for the treatment of malaria (*Table 6*) (Buckner et al., 2012).

Translational			Development	
Preclinical	Phase I	Phase II	Phase III	Approved
Mefloquine	CRDI 97/98 (trioxolane)	Artemisone	Eurartesim (Dihydroartemisinin + piperaquine)	Coartem-D (Artemisinin + lumefantrine)
Mirincamycin (*P. vivax*)	AQ13	Artesunate + Ferroquine	Pyramax (Pyronaridine + artemisinin)	Amodiaquine + artemsinin (Coarsucam)
Trioxaquine (trioxane + quinoline)	Isoquine	OZ277 (trioxolane) + piperaquine	Artemisinine + mefloquine	
Pyridones (ETC)	Tafenoquine (*P. vivax*)	Methylene bleu + amodiaquine	Azithromycin + chloroquine (AZCQ)	
MK 4815	4-Pyridone (ETC)	Fosfodomycine + clindamycin	Trimethoprim/sulphamethoxazole (Co-trimoxazole)	
2 Novartis compounds	Actelion antimalarial	Tinidazole (nitroimidazole) *P. vivax*	Arterolane maleate + piperaquine phosphate	
GNF156		SAR97276 (choline analog)		
Oxaboroles		Methylene blue, chloroquine		
DSM265		Fosmidomycin, clindamycin (Foscilin)		
P218		SSR97193, artesunate (Ferroquine)		
Genz668764		AN3661		
RKA 182		OZ439 (trioxolane)		

Table 6 – Some of the new antimalarials under development, status in 2009. *Source:* (Olliaro and Wells, 2009) http://www.malariajournal.com/content/11/1/316/table/T4

I.5 – Artemisinins

I.5.1 – Artemisinin antimalarial class

Artemisinin belongs to a unique class of antimalarial drugs. They are sesquiterpene trioxane lactones, and its high antimalarial activity is due to the presence of an endoperoxide bridge (Figure 11 A) (White, 2008b). Artemisinin (or Qinghaosu) was discovered by Dr. Youyou Tu and her team under Project 523 of the Chinese government created to help North Vietnamese fight malaria during the war against the United States. From Chinese traditional medicine a candidate stood out: the Chinese herb Qing Hao or sweet wormwood (*Artemisia annua,* Figure 11 B), that was used for many centuries to cure several illnesses such as high fevers (Cui and Su, 2009; Hsu, 2006a, 2006b; Klayman, 1985).

Figure 11 - Artemisinin and Artemisia annua.

A- *Chemical structure of artemisinin, the red arrow indicates the endoperoxide bridge;* ***B –*** *Pictures of Artemisia annua plant, from which the natural artemisinin molecule was isolated.* (Dondorp et al., 2010). *http://crdp.ac-besancon.fr/. <photo credit: Action Me: Brigitte Betzelt.*

Since 2001 the WHO recommends the use of artemisinin-based combination therapies (ACTs) as the first line treatment for uncomplicated malaria. Because artemisinin has poor bioavailability limiting its efficiency, that it is soluble neither in water nor in oil and thus cannot be orally administrated (Kamchonwongpaisan and Meshnick, 1996), several semi-synthetic derivatives have been created (Figure 6). The most widespread semi-synthetic derivatives of artemisinin are: artesunate, artemether, β-arteether, and dihydroartemisinin. Dihydroartemisinin is the bioactive metabolite of all artemisinin derivatives and the most potent derivative; however, it has the shortest half-life and seems to be more toxic for mouse neuroblastoma *in vitro* (Schmuck and Haynes, 2000; Wesche et al., 1994). Deoxyartemisinin is a biologically inactive derivative in which the endoperoxide bridge was replaced by a single oxygen (Avery et al., 1993). Artemether and artesunate have better bioavailability than artemisinin and are used clinically in artemisinin combination therapy. Arteether (also known as artemotil) is less frequently used. Artelinic acid is an experimental semi-synthetic artemisinin derivative, that presents lower neurotoxicity than the arteether and artemether (Genovese et al., 2000) but is three times-fold more toxic than artesunate (Li et al., 2007). This derivative is unlikely to be commercially available, because it offers no clear benefits over the artemisinins already available (artesunate and artemether). Since 2010 these derivatives can be produced with a high-yield procedure in yeast for a stable industrial supply of these antimalarials for the developing countries, with a reduced average cost (Paddon et al., 2013).

Artemisinins are also effective on other pathogens such as *Schistosoma spp.,* opportunistic *cytomegalovirus, Trypanosoma cruzi,* and *Trypanosoma brucei rhodensiense* (Mishina et al., 2007) and *Leishmania spp* parasites (Avery et al., 2003; Yang and Liew, 1993), at a micromolar range for intracellular stages. They accumulate preferentially in infected erythrocytes (Gu et al., 1984; Vyas et al., 2002) , and locate mostly in parasites and their neutral lipids (Hartwig et al., 2009; Olliaro and Wells, 2009).

Artemisinins are highly potent and fast acting drugs, though they have a short plasma half life. They are active against the broadest range of *Plasmodium* life-cycle stages (Figure 8), act rapidly on asexual blood stages of CQ sensitive and resistant strains of *P. falciparum* and *P. vivax*, and are also efficient against gametocytes (sexual stages) (Chen et al., 1994; Dutta et al., 1989; Golenser et al., 2006; Kamchonwongpaisan and Meshnick, 1996; Kombila et al., 1997; Kumar and Zheng, 1990; Mehra and Bhasin, 1993; Targett et al., 2001; Ter Kuile et al., 1993). However, artemisinin does not possess sporoncidal activity (against sporozoites) or prophylactic activity (against preerythrocytic stages). Because of their short plasma half-life, they cannot be used in prophylaxis (Kamchonwongpaisan and Meshnick, 1996). They are safe, with little side effects, and inexpensive (Dondorp et al., 2010; Grimberg and Mehlotra, 2011). Alone they are not sufficient to clear the organism of parasites. Although the parasitemia often drops underneath detection, when monotherapy with artemisinin is stopped, the few remaining parasites, that are the most resistant, will proliferate again (Maude et al., 2009). This is dangerous for the infected individual and can cause emergence of artemisinin resistance. For this, WHO recommends the use of artemisinin in combination with a long half-life drug (ACTs).

I.5.2 – Artemisinin mode of action

The mechanism of action of these drugs is very controversial and remains uncertain but several hypotheses have been formulated (Grimberg and Mehlotra, 2011). The knowledge of artemisinin's target is highly important to be able to predict and counter emerging resistances to this drug. This target could be used to find new inhibitors or other artemisinin derivatives active against resistant parasites. Many potential mechanisms have been suggested. Artemisinin has been called the "magic bullet" by X. C. Ding and coworkers, as it has been described to act in many different ways (Ding et al., 2011) (Figure 12). Several different models, not all mutually exclusive, have been suggested and will be presented below.

I.5.2.1 - Free radical generation

Free radicals such as reactive oxygen species are toxic and cause damage to cells. These are more prone to appear in presence of haeme or iron (Halliwell and Gutteridge, 1990a, 1990b; Kamchonwongpaisan and Meshnick, 1996), but may also result from interference with ETC (Krungkrai, 2004). Artemisinin via the Endoperoxide Bridge would be able to generate such radicals and hence be toxic for the parasite.

I.5.2.2 - Interference with haeme detoxification pathway

Plasmodium, during blood stages, degradates hemoglobin in the digestive vacuole from the infected erythrocyte, as a source of amino acids (Figure 12). As free haeme is toxic for the parasite, a mechanism of crystallization into free haemozoin takes place. It has been proposed, that the endoperoxide bond, when cleaved by haeme in presence of ferrous ion (Fe^{2+}), produces free radicals

of artemisinin. These free radicals would interfere with the process of free haeme detoxification by the parasite (Meshnick et al., 1993; Meunier and Robert, 2010). Artemisinin activity was shown to be dependent on haemoglobin uptake and digestion by the parasite (Klonis et al., 2011). This hypothesis arose from the fact that artemisinin is activated by high Fe^{2+} free ions concentrations (Meshnick et al., 1993), and that artemisinin reacts *in vitro* and *in vivo* with haeme (Pandey et al., 1999; Robert et al., 2005). Thus iron is required for artemisinin antimalarial activity (Kamchonwongpaisan and Meshnick, 1996; Meshnick et al., 1993, 1991), and probably haeme too (Golenser et al., 2006; Wei and Sadrzadeh, 1994).

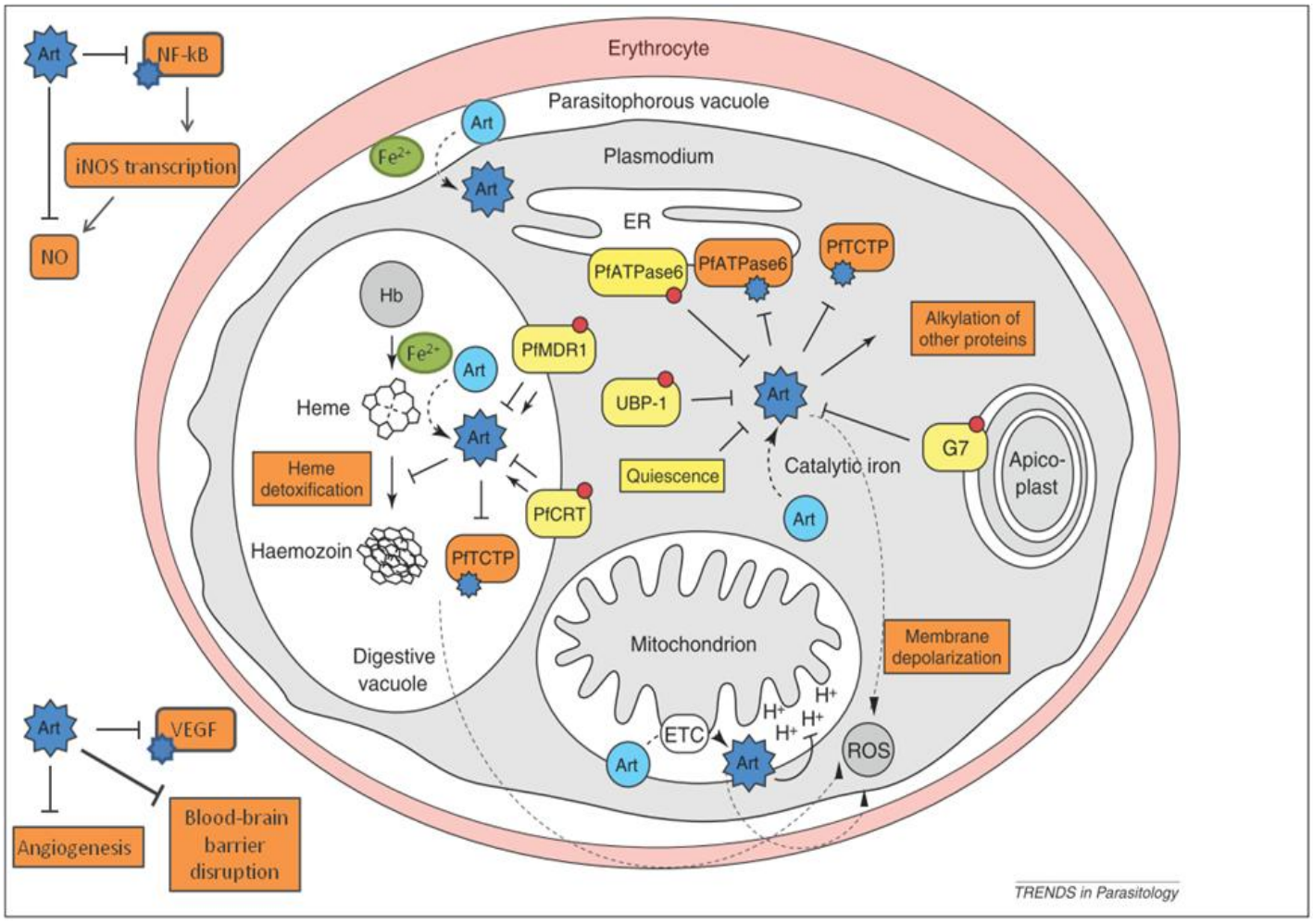

Figure 12 - Potential mode of action of artemisinin.

Proteins in which mutations (red dots) have been shown to increase (arrow) or to decrease (inhibition line) in vitro sensitivity to ART are indicated. Putative targets or target mechanisms are labeled in orange and putative resistance factors in yellow. Art – Artemisinin; ETC – Electron Transport Chain; ER – Endoplasmic Reticulum; G7 – putative apicoplast transporter; Hb – Hemoglobin; iNOS - nitric oxide synthase; NF-kB – nuclear factor kappa B; NO - nitric oxide; PfATPase 6 – P. falciparum endoplasmic reticulum Ca^{2+} ATPase; PfCRT – P. falciparum Chloroquin Resistance Transporter; PfMDR1 – P. falciparum Multi Drug Resistance protein 1; PfTCTP - Transitionally controlled tumor protein; ROS – Reactive Oxygen Species; UBP-1 - putative de-ubiquitinating enzyme; VEGF - vascular endothelial growth factor. Adapted from (Ding et al., 2011).

I.5.2.3 - Protein alkylation

Due to its reactivity, artemisinin interacts with nearby molecules such as proteins (Asawamahasakda et al., 1994a, 1994b, 1994c; Kamchonwongpaisan and Meshnick, 1996; Yang et al., 1993), and haeme (Hong et al., 1994; Meshnick et al., 1991) but not with DNA (Aboul-Enein, 1989; Yang et al., 1994).

Some proteins were found to covalently react with artemisinin and derivatives. Transitionally controlled tumor protein (PfTCTP) was found to interact with radiolabeled dihydroartemisinin, as detected by immunoprecipitation experiments (Bhisutthibhan and Meshnick, 2001; Bhisutthibhan et al., 1998). There is still a lack of functional information for PfTCTP (Krishna et al., 2006; Li and Zhou, 2010), however a slight increase in copies number of this protein was observed in *P. yoelli* artemisinin-resistance strain (Walker et al., 2000). In 2013, PfTCTP was crystallized and putative binding sites for artemisinin were described (Eichhorn et al., 2013).

I.5.2.4 - Interference with the electron transport chain of mitochondria

The electron transport chain is the parasite's key mechanism to produce ATP. The mitochondrial genome is 6Kb long and is composed of three genes (*cytochrome b, COXI, COXIII*). Administration of artemisinin to *Macaca assamensis* monkeys infected with *P. inuii* resulted in parasite mitochondria swelling (Jiang et al., 1985; Li et al., 2005). Growth of yeast on non fermentable carbon sources in presence of artemisinin, strongly inhibited yeast growth (Li et al., 2005). Artemisinins also affects the electron transport chain in neuronal cell culture (Schmuck et al., 2002).

Artemisinin and primaquine were shown to inhibit the respiratory chain, by inhibiting mitochondrial inner membrane potential, of asexual and sexual stages *P. falciparum* (Krungkrai, 2004; Krungkrai et al., 1999). Inhibiting the electron donor NADH dehydrogenase in the electron transfer chain (ETC) antagonizes the action of artemisinin. Deletion of *nde1* and *ndi1* (genes encoding for NADH dehydrogenase in *Plasmodium*) and *nde2* (gene encoding for NADH dehydrogenase in yeast), confered resistance to artemisinin, while overexpression of these genes increases sensitivity to artemisinin, activated by iron (Li et al., 2005). It was suggested that artemisinins would disrupt the ETC and depolarize the mitochondria membrane, producing ROS (Reactive Oxygen Species) that lead to parasite death (Figure 12). This depolarization was also verified on isolated mitochondria (Wang et al., 2010). Deoxyartemisinin lacks an endoperoxide bridge and has no effect on ROS production, though distant molecules (OZ209), presenting this peroxide bridge, have an effect on this production.

I.5.2.5 - Interference with the immune system

Like other sesquiterpene lactones, artemisinin would seem to inhibit the activation of the nuclear factor NF-kB, and hence inhibit the transcription of nitric oxide synthase (iNOS). iNOS is essential for the production of nitric oxide (NO) that plays a fundamental role in inflammation and immune response. NO derivatives have been shown to be present in high plasmic levels in patients infected with *P. falciparum* and *P. vivax* and are related to the severity of the disease, like neuronal dysfunction (Clark et al., 1991; Nussler et al., 1994). This way artemisinin would have antiparasite and neurological therapeutic effects in complicated malaria cases. By suppressing NF-kB and therefore NO production, artemisininin reduces considerably the burden of the disease (Aldieri et al., 2003). This is a path worth exploring for further antimalarials.

Artemisinin has been shown to cause p53 dependent apoptosis in cancer cell lines, mediated by mitochondrial pathways (Wu et al 2004, Disbrow et al 2005, Singh and Lai 2004). Artemisinin could be able to induce apoptosis or directly kill cancer cells by affecting calcium homeostasis, as shown with calcium sensitive dyes (de Pilla Varotti et al., 2008; Riganti et al., 2009; Stockwin et al., 2009).

I.5.2.6 - Effect on angiogenesis

Artemisinin is thought to inhibit vascular endothelial growth factor (VEGF) and hence suppress angiogenesis. Dihydroartemisinin would inhibit the binding of VEGF to its receptor as well as inhibiting the expression of this receptor, in human umbilical vein endothelial cells (HUVEC) (Chen et al., 2004, 2003). It is known that in severe and cerebral malaria, endothelial cell activation as well as the disruption of blood-brain barrier occurs (Thumwood et al., 1988). VEGF activation induces blood-brain barrier disruption and also angiogenesis, where parasite sequestration [7] has occurred, resulting in brain edema and hemorrhages, leading to long lasting brain damages (Deininger et al., 2003). VEGF regulation is also an important path to consider in new antimalarial research for the reduction of malaria burden.

I.5.2.7 - Inhibition of the sarco/endoplasmic reticulum calcium ATPase (PfATP6)

PfATP6 is the SERCA (sarco/endoplasmic reticulum calcium ATPase) of *P. falciparum*. In 2003, Krishna and collaborators suggested that PfATP6 was the direct target of artemisinins by heterologously expressing this protein in *Xenopus laevis* oocytes membrane, though no proof of protein expression was shown (Eckstein-Ludwig et al., 2003). Later the single mutation L263E was described to render PfATP6 insensitive to artemisinin (Uhlemann et al., 2005). Recombinant *P. falciparum* lines with the mutation L263E showed a marginal non-significant tendency to decrease *in vitro* sensitivity to artemisinin (Valderramos et al., 2010).

Later, the same group found in artemisinin-resistant field isolates, the PfATP6 mutation S769N (Jambou et al., 2005). By allelic exchange technology, a group managed to insert the S769N mutation into *P. falciparum* 3D7 strain to determine if this mutation was modulating a decrease in artemisinin sensitivity (Cui et al., 2012). This strain did not present a higher artemisinin IC_{50} than the wild type parasites, as well as no altered expression of *pfatp6* mRNA. Hence, the modification S769N does not seem to be at the origin of artemisinin resistance (Cui et al., 2012). There is no clear evidence of correlation between *pfatp6* mutation L263E nor S769N and artemisinin resistance.

In *Toxoplasma gondii,* another *Apicomplexa* parasite, thapsigargin and artemisinin were reported to induce elevated cytoplasmic calcium by inhibiting the TgSERCA protein, localized in the endoplasmic reticulum of the parasite (Nagamune et al., 2007a). Artemisinin triggered an altered intracellular calcium concentration and a calcium-dependent secretion of cellular invasion proteins (Nagamune and Sibley, 2006; Nagamune et al., 2008, 2007a, 2007b). They managed to clone TgSERCA and showed that this protein was sufficient to restore calcium transport in a calcium-ATPase defective yeast strain, and that it was inhibited by both thapsigargin and artemisinin. The same authors, created *in vitro* three artemisinin resistant *Toxoplasma gondii* clones, by chemical mutagenesis

[7] Sequestration – adherence of *Plasmodium* infected eryhtrocytes to vascular endothelium (David et al 1983 PNAS)

(Nagamune et al., 2007a). These clones were reported to possess an altered calcium homeostasis (Nagamune et al., 2007a). When the resistant strains were treated with 10 µM artemisinin, there was no increase of intracellular calcium concentration (Nagamune et al., 2007a).

Artemisinin and amino-alcools antimalarials were reported to inhibit SERCA extracted from rabbit muscle (SR) at periphysiological concentrations (Toovey et al., 2008), inhibited by dihydroartemisinin (IC_{50} – 1 µM) but with an activatory effect at 10 µM, which makes these results quite dubious (Toovey et al., 2008). These results were obtained with wild type SR (without the mutation E255L, see below), eventhough SERCA1a was previously reported to be insensitive to artemisinin by the same group of S. Krishna (Eckstein-Ludwig et al., 2003; Uhlemann et al., 2005).

Altogether, although some reports would indicate changes in calcium homeostasis, no direct link between PfATP6 or homologous proteins and artemisinin was unambiguously described.

In 2010, PfATP6 was heterologously expressed in yeast and purified (Cardi et al., 2010b). ATPase activities showed that PfATP6 was inhibited by classical SERCA inhibitors (thapsigargin, cyclopiazonic acid – CPA, and 2,5-Di-t-butyl-1,4-benzohydroquinone – BHQ) but not by artemisinin and derivatives. These experiments brought important evidences that PfATP6 was not the direct target of artemisinins.

I.5.3 - Artemisinin resistance

Resistance is suspected when there is an increase in parasite clearance time by more than 10%, and on day 3 of treatment parasitemia is still detectable. Resistance is confirmed when an artemisinin-based monotherapy fails, with persistence of parasites after 7 days, or recrudescence after 3 days after parasite clearance (Dondorp and Ringwald, 2013).

Higher recrudescence after artemisinin treatment has been reported for the first time in 2004 in Cambodia (Denis et al., 2006; Dondorp et al., 2010; World Health Organization, 2007).

Artemisinin resistance and its definition is still a work in progress (Fairhurst et al., 2012) and some researchers claim that we should not mention resistance, and that treatment failure is not synonym of resistance (Krishna and Kremsner, 2013). The term “treatment failure of artemisinin combination therapy” (TFACT) has been suggested to be used instead of “artemisinin resistance”, and that this phenomenon should not be exaggerated by public health professionals. However ignoring an artemisinin resistance emergence can put in danger the urgency of finding early solutions when it spreads (Dondorp and Ringwald, 2013). If an ACT partner fails (*e.g.,* artemisinin), the role of killing the little remaining parasites is left to the second partner, making treatment entirely dependent from the second partner and, in a resistance background to antimalarials, clearance time can be longer and ACT failure can develop. Artemisinin resistance is partial in the Greater Mekong Subregion, and waiting for the term TFACT to be established, which is just a question of semantics, would only delay the important decisions and actions, risking the spread of resistant parasites to regions of great endemicity such as Sub-Saharan Africa (Dondorp and Ringwald, 2013).

Cambodia was one of the first countries to adopt the ACTs in 2001, but artemisinin monotherapy was already available since the 70's in the private sector, and served 78% of all treatments (Yeung et al., 2008). Counterfeit and non-authorized antimalarials (inappropriate concentration of ingredients, contamination with other drugs, impurities, poor quality, stability or packaging) are the main cause for the artemisinin resistance emergence (Newton et al., 2003). But also an exceptional drug pressure in the region, suboptimal time of therapy, together with low malaria transmission in the area, allowed the establishment of a population of resistant parasites, but perhaps host factors or a unique parasite genetic background may also have contributed (Dondorp et al., 2010; Maude et al., 2009; Pongtavornpinyo et al., 2008; Rathod et al., 1997; White and Pongtavornpinyo, 2003). Cambodia has also been the starting point of chloroquine resistance (Verdrager, 1986). Atovaquone-proguanil is the alternative combination for artemisinin; however the prohibitive high price renders this option inaccessible in low-income countries. Triple-therapy – administration of three drugs together - and multiple first-line treatments (MFTs)[8] can be strategies used to avoid artemisinin resistance spread. Some new combinations with artemisinin are being studied. Mass drug administration to the population regardless of infection or the disease stage, has been used in the past to control malaria, minimizing drug pressure. However there is always a part of the population that refuses to participate and the logistic organization is huge. These initiatives have reduced parasite and disease prevalence but were not successful in preventing transmission (Dondorp et al., 2010; von Seidlein and Greenwood, 2003).

Artemisinin resistance has been induced *in vitro* on *P. falciparum* and *in vivo* in a murine model of malaria (Afonso et al., 2006), but also *in vitro* from field isolates from French Guyana (Jambou et al., 2005) and central Africa (Menard et al., 2005). Artemisinin increased tolerance has been proposed to be linked to quiescence mechanisms (previously described in other *Plasmodium* species and pathogenic organisms). A group of researchers has obtained *in vitro* an artemisinin resistant strain, induced by long-term (5 years) drug selective pressure (Witkowski et al., 2013). When under drug pressure, this strain (F32-ART5) arrests its cell cycle at the ring stage, surviving high artemisinin doses. This quiescence phenomenon is stopped after drug removal and the parasite's developmental cycle continues normally.

I.5.4 -Molecular markers of artemisinin resistance

The scientific community is desperately in search of a molecular marker that could detect the presence of an artemisinin resistant strain. Following the multi-target theory of artemisinin action it would be necessary that mutations arise in all targets before a real artemisinin resistance appears, and not only sensitivity reduction. Global Plan for Artemisinin Resistance Containment (GPARC) has a major goal for determining the molecular markers underlying artemisinin resistance. This initiative is complicated to achieve *in vitro* and thus researchers have relied on field isolates epidemiology (Bright and Winzeler, 2013). WHO has initiated a containment program for Western Cambodia and Thailand that involves early diagnosis and treatment programs, banning artemisinin monotherapy in

[8] Multiple first-line treatment (MFT) – instead of only using one official first-line treatment (such as ACTs), the implementation of several first-line official therapies will enable the lowering of alternative side treatments used nowadays, that contribute for resistance and counterfeit.

the private sector, increasing the use of impregnated mosquito nets and following the migrations from the country (Amaratunga et al., 2012; Phyo et al., 2012).

A molecular marker has to fulfill several requirements: i) progressive loss of wild-type allele in resistance areas, ii) higher frequency of the mutant alleles in the area of resistance to artemisinin, iii) mutation localized in the DNA region of positive selective pressure from artemisinin treatment, iv) it has to be a non-synonymous mutation, in a non-naturally polymorphic region (Ariey et al., 2014).

I.5.4.1 - Candidate gene sequencing

Studies are very controversial about molecular markers for artemisinin resistance. Some studies find specific mutation associated to resistance and others don't find any relation with the same mutation, in the same genes or regions. Here we list some of these genes and some of the corresponding mutations described to confer artemisinin resistance (Table 7). These studies have been made by sequencing specific target genes in field isolates sensitive vs. resistant to artemisinins and derivatives, or in induced artemisinin-resitant laboratory strains.

Candidate genes proposed to be involved with artemisinin resistance: (Price et al., 2004)

- *Pfmdr1, the P. falciparum multidrug resistance gene 1, encodes for the P-glycoprotein homologue 1 (Pgh1), an ATP-binding cassette transporter (Anderson et al 2006);*
- *Pfcrt, the P. falciparum chloroquine resistance transporter,*
- *Pfhpt, P. falciparum hypoxanthine phosphoribosyl transferase , involved in purine biosynthesis*
- *Pftctp, P. falciparum translational controlled tumor protein (TCTP) has been found to bind artemisinin and be related to resistance in P. yoelii*
- *Pfubp-1 (putative de-ubiquitinating enzyme) encodes for a deubiquitinating enzyme*
- *Pf COX-III, P. falciparum Cytochrome c oxydase 3*
- *Pfdhps and Pfdhfr, P. falciparum dihydrofolate reductase (pfdhfr) and dihydropteroate synthase (pfdhps)*
- *Pf10_0026 (PArt), encoding a hypothetical protein of unknown function*
- *PF3D7_1343700 kelch propeller domain (K13-propeller), unknown function*
- *Pfmrp1, P. falciparum Multidrug Resistance Protein 1*
- *Pfatp6 , the endoplasmic reticulum calcium ATPase (SERCA) of P. falciparum*

	Associated with artemisinin or ACT resistant field isolates		*Associated with ex vivo or in vivo artemisinin resistant strains*	
Gene	Yes	No	Yes	No
Pfmdr1	(Alker et al., 2007; Borges et al., 2011; Chen et al., 2010; Duraisingh, Roper, Walliker, & Warhurst, 2000; Golenser, Waknine, Krugliak, Hunt, & Grau, 2006; Preechapornkul et al., 2009; Sidhu et al., 2006;Phompradit et al., 2011, Chavchich et al., 2010;Price et al., 2004; Shahinas et al., 2010)	(Dondorp et al., 2009; Noedl et al., 2008 ; Jambou et al., 2005; Kwansa-Bentum et al., 2011; Imwong et al., 2010)		(Afonso et al., 2006; Ariey et al., 2014; Witkowski et al., 2013)
	(Duraisingh et al., 2000; Anderson et al., 2005; Shahinas et al., 2010; Sisowath et al., 2005; Veiga et al., 2011)	(Chavchich et al., 2010; Dondorp et al., 2009; Imwong et al., 2010; Kwansa-Bentum et al., 2011; Preechapornkul et al., 2009)		(Afonso et al., 2006; Ariey et al., 2014; Witkowski et al., 2013)
Pfmrp1	(Veiga et al., 2011)			(Ariey et al., 2014;)
Pfcrt	(Bacon et al., 2009; Sidhu et al., 2006, 2002)	(Chavchich et al., 2010; Kwansa-Bentum et al., 2011)		(Afonso et al., 2006; Ariey et al., 2014)
HSP70			(Natalang et al., 2008; Witkowski et al., 2010)	
Pfhpt			(Natalang et al., 2008; Witkowski et al., 2010)	
Pftctp		(Chavchich et al., 2010; Jambou et al., 2005; Kwansa-Bentum et al., 2011)		(Afonso et al., 2006; Ariey et al., 2014; Walker et al., 2000)
Pfubp1		(Imwong et al., 2010)	(Hunt et al., 2010, 2007)	(Chavchich et al., 2010)
COX-III		(Imwong et al., 2010)		
Pfdhps and Pfdhfr	(Bacon et al., 2009)			
PfATP6	S769N and A623E (Jambou et al., 2005; Pillai et al., 2012; Shahinas et al., 2010) Ferreira et al., 2007	(Adhin et al., 2012; Bacon et al., 2009; Chavchich et al., 2010; Cojean et al., 2006; Dahlström et al., 2008; Dondorp et al., 2009; Huang et al., 2012; Imwong et al., 2010; Kamugisha et al., 2012; Kwansa-Bentum et al., 2011; Menemedengue et al., 2011; Miao et al., 2013; Noedl et al., 2008; Phompradit et al., 2011; Tahar et al., 2009; Zhang et al., 2008)		*P. chabaudi:* (Afonso et al., 2006; Witkowski et al., 2010) 3D7 *P. Falciparum* strain PfATP6_S769N: (Cui et al., 2012)
Pf10_0026 *PArt*	(Deplaine et al., 2011)			

Table 7 - Some of the most frequently associated candidate gene with artemisinin resistance.

Enumeration of some studies that found/did not find association between mutations in certain genes and artemisinin or ACT resistances. ACT – Artemisinin Combination Therapies; Pfmdr1, Plasmodium falciparum multidrug resistance gene 1; Pfcrt, the Plasmodium falciparum chloroquine resistance transporter; Pfhpt, P. falciparum hypoxanthine phosphoribosyl transferase; Pftctp, P. falciparum Translational controlled tumor protein (TCTP); Pfubp-1, putative de-ubiquitinating enzyme; Pf COX-III, P. falciparum Cytochrome c oxydase 3; Pfdhps and Pfdhfr, P. falciparum dihydrofolate reductase (pfdhfr) and dihydropteroate synthase (pfdhps); Pf10_0026 (PArt); PF3D7_1343700 kelch propeller domain (K13-propeller); Pfmrp1, P. falciparum Multidrug Resistance Protein 1; Pfatp6, the P. falciparum SERCA.

I.5.4.2 - Genome wide screening field artemisinin resistance population

Some research groups have analyzed the entire genome, instead of just point mutations in candidate genes, of field isolated parasites from regions were ACTs resistance have been reported, or from laboratory induced artemisinin-resistant clones.

Miotto and coworkers have found a genomic region in three genetically distinct resistant field parasite populations of a restricted geographic area (West Africa and Southeast Asia) (Miotto et al., 2013b). This population genetics study highlighted genomic regions that are significantly associated with delayed parasite clearance after artemisinin treatment located on chromosome 13 (also identified in other studies (Cheeseman et al., 2012; Miotto et al., 2013a; Takala-Harrison et al., 2013)). The genes are localized in this region are: *pfcrt, pfmdr1, pfdhps, pfdhfr pfatp6,* and other synonymous mutations (Miotto et al., 2013b).

Another study was performed in three southeast regions: Wang Pha (Thai – Burmanese border, where moderately delayed parasite clearance after artesunate-mefloquine treatment was reported); Pailin and Tasanh (Cambodia, suspected emerging artemisinin resistance); and in Bandarban (Bangladesh, where artemisinin is poorly used). DNA was used from field isolates and genotyped (Takala-Harrison et al., 2013). Regions under artemisinin positive selective pressure were found: chromosome 10, 13 and 14. Chromosome 13 (two significant SNPs) seems to be in a region that suffered recent strong positive selection in Cambodia, chromosome 10 (one SNP) and 14 (one SNP) are localized in or near genes involved in post-replication DNA repair (Takala-Harrison et al., 2013). These SNPs are localized in or near genes involved in the same metabolic pathway as described by Cheeseman and collaborators in 2012 (Cheeseman et al., 2012). 2.4% of *P. falciparum* genome was found to be under artemisinin positive selection and three genes were identified: *pfcrt, pfdhps* and *pfdhfr.* Three regions on chromosome 6, 13 and 14 were found to have evidences for positive selection. No evidences of selection of the genes: *pfatp6* or *Part* was found (Cheeseman et al., 2012).

A recent study used whole-genome sequencing of an artemisinin resistant parasite line from Africa selected *in vitro* (F32-ART5 (Witkowski et al., 2013) and field isolates from Cambodia. A new region was reported to be linked with artemisinin resistance *in vitro* and *in vivo*: the *PF3D7_1343700* kelch propeller domain (K13-propeller) (Ariey et al., 2014). The function of this region is still unclear, but it could have a role in protein-protein interactions. 8 mutations in 7 genes were found in the artemisinin resistant line but only one of these genes has been previously reported to be associated

with artemisinin low sensitivity *in vitro*: the cysteine protease falcipain 2a (Klonis et al., 2011). By exploring the whole-genome sequences following the artemisinin pressure cycle two mutations were found to appear first: D56V and M476I and remained stable after, and were sufficient to confer artemisinin resistance. Four mutant alleles were also described to carry a single non-synonymous SNP within a kelch repeat of the C-terminal K13-propeller domain: Y493H, R539T, I543T and C580Y. Mutation C580Y has been rapidly increasing in frequency in field isolates from Pailin and Battambang (Cambodia) since 2001 to 2012, and parasites carrying this mutation have a significantly higher clearance time when compared to the other mutations. Isolates from Pursat and Ratanakiri were tested for parasite clearance half-lives and mutation, confirming that K13-propeller polymorphisms are a good marker of clinical artemisinin resistance. None of the previously mutations reported to be associated to artemisinin resistance were found in the genes *pfcrt, pftctp*, *pfmdr1, pfmrp1, pfatp6, pfubpcth – P. chabaudi ubp1* orthologue, and *pfmdr1* (Ariey et al., 2014).

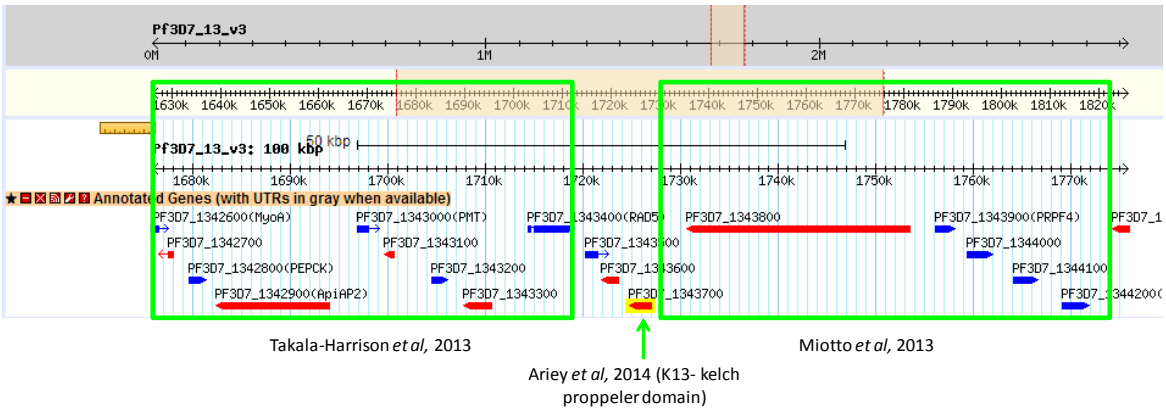

Figure 13 - Genomic region of P. falciparum 3D7 strain in chromosome 13 that was highlighted to be associated with artemisinin resistances.

These studies performed genome wide screening studies in field artemisinin resistant popultions vs. sensitive. Genomic regions with mutations associated with artemisinin insensitivity found in (Ariey et al., 2014; Miotto et al., 2013b; Takala-Harrison et al., 2013)*. On top of the image we have rullers that indicates the landmark of the genomic region of 3D7 P. falciparum we are looking at in PlasmoDB. The arrows indicate annotated genes coding for known or putative protein functions, in blue the genes oriented on the DNA forward strand, in red the genes oriented on the reverse DNA strand. (http://plasmodb.org/plasmo/).*

Figure 13 shows a summary of *P. falciparum* genomic regions found associated with loss of artemisinin sensitivity, after genome wide screening of resistant parasites. With this figure we can see that all three studies (Ariey et al., 2014; Miotto et al., 2013b; Takala-Harrison et al., 2013) focus on chromosome 13. It is important to note that both Cheeseman et al and Takal-Harrison et al described the flanking regions, but never the region described by the most recent study (Ariey et al., 2014).

All of these studies highlight different mechanisms for artemisinin mode of action and resistance mechanisms. This remains a very controversial subject and it is still unclear how this antimalarial acts and if either it has a single target or a multitude of mechanisms that would be concordant with a “magic bullet” theory (Ding et al., 2011).

II – *Plasmodium* Membrane Transporters as Potential Antimalarial Targets

II.1 - Malaria Membrane Transport Proteins

The malaria infected erythrocyte is composed of a multitude of membranes and cellular compartments. The parasite expresses several transmembranar proteins that play key roles in moving solutes into and between the parasite and the erythrocyte membrane, and these proteins are also involved in resistance mechanisms. These membranes are: the infected erythrocyte plasma membrane; the parasite plasma membrane; the parasite's nucleus; the parasite's endoplasmic reticulum; the parasite's Golgi apparatus; the parasitophorous vacuolar membrane; the large acidic digestive vacuole and the smaller acidocalcisomes; the parasite's mitochondrial double membrane; and finally the apicoplast limited by four membranes (Figure 14). As the parasite is not in direct contact with the erythrocyte cytosol, the only way to ensure essential ion and nutrients uptake as well as evacuation of metabolic waste, is by tightly regulating the localization of pumps. P-type ATPases pumps are crucial to perform this role. *P. falciparum* genome encodes for 13 predicted P-type ATPases (Martin et al., 2009).

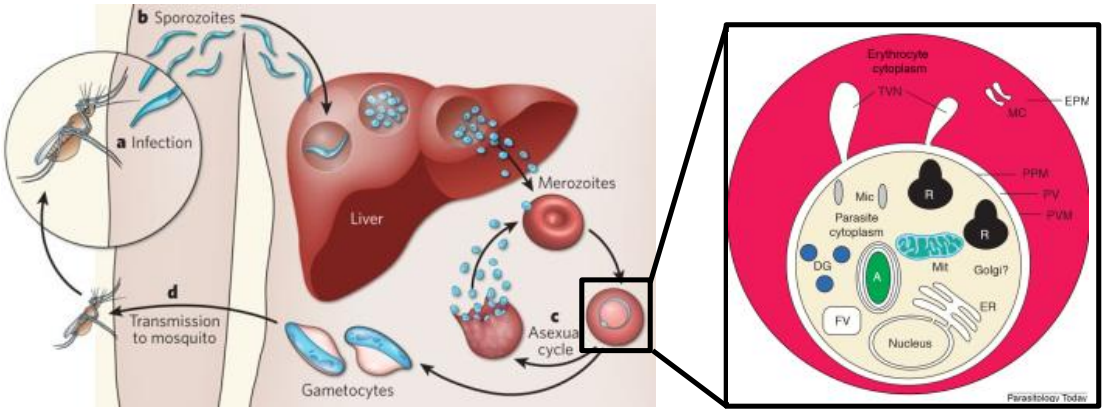

Figure 14 - Schematic representation of the infected erythrocyte with the membranar complexes highlighted.

A (apicoplast); DG (dense granules) [9]; EPM (erythrocyte plasma membrane); ER (Endoplasmic reticulum); FV (food vacuole) [10]; MC (Maurer's clefts) [11]; Mic (micronemes)[12]; Mit (mitochondrion); PPM (parasite plasma membrane); PV (parasitophorous vacuole) [13]; PVM (parasitophorous vacuolar membrane); R (rhoptries) [14]; TVN (tubulovesicular network) [15]. (Van Dooren et al., 2000) *http://sitn.hms.harvard.edu/flash/2013/1001-bites-the-road-to-a-successful-malaria-vaccine/.*

[9] Dense granules – they are secretory organelles.
[10] Food vacuole – or digestive vacuole, is the site where haemoglobin is degradated during *Plasmodium* erythrocytic stages.
[11] Maurer's clefts – they might play a role as an intermediate compartment in protein transport across the parasite and erythrocyte cytoplasm.
[12] Micronemes – such as rhoptries, are secretory organelles that play a role in gliding motility and invasion.
[13] Parasitphorous vacuole – when the parasite invades the host-cell, the invagination into the cell creates a compartment called the parasitophorous vacuole, which is dellimited by the host-cell plasma membrane.
[14] Rhoptries – is a secretory organelle that may contain several enzymes that are released during host-cell invasion. They are characteristic of motile stages of Apicomplexa

Passed 12h to 18h post-invasion of the red blood cell by the parasite, an increase in permeability of the erythrocyte takes place by upper expression of host and parasites channels and pumps, to enable entry of parasite's essential nutrients, ion homeostasis regulation, and mediate efflux of metabolic waste (e.g., lactic acid) (Becker and Kirk, 2004; Grellier et al., 2012; Martin et al., 2009; Staines et al., 2010). These newly established routes are called "new permeability pathways" (NPPs), and represent a unique class of ion selective channels (Baumeister et al., 2006; Becker and Kirk, 2004; Kirk et al., 1994). Some of these channels are thought to be host erythrocytes proteins, activated by the parasite. Solutes are considered to pass freely between the erythrocyte and the parasitiphorous vacuole, via low selectivity and high capacity channels (Desai et al., 1993); however the movement into the parasite is more tightly regulated by channels, transporters and pumps.

Some transporters are already known to play an important role in antimalarial resistance or drug sensitivity: the *P. falciparum* chloroquine resistance transporter (PfCRT, member of the drug/metabolite superfamily); the *P. falciparum* multidrug resistance protein (PfMRP), *P. falciparum* Na^+/H^+ exchanger (PfNHE), and the P-glycoprotein homologue 1 (Pgh1, coded by *pfmdr1* gene is a member of the ABC transporter family).

To date 100 known and putative transporter sequences have been annotated in the *P. falciparum* genome (Martin et al., 2005). This collection has been named the *Plasmodium* "permeome" (Figure 15) (Martin et al., 2009, 2005; Staines et al., 2010). Transporters are widely studied as potential drug targets. The cellular localization of some of these transporters is known, but membrane proteins are difficult to localize by immune-fluorescence techniques because an important part of the protein is imbedded in the membrane and hence not apparent and the extracellular part is not always accessible. Another option is to couple a GFP (epitope tagged or fluorescence recombinant protein) to the gene encoding the protein; this construction is transfected into parasites and the fluorescent protein can be visualized by fluorescence microscopy. In Figure 15 some confirmed (proteins annotated with a yellow dot) and predicted localization of *P. falciparum* membrane transporters are shown (Martin et al., 2009) .

II.2 - Transporters as Potential Drug Targets

For their implication in important physiological roles and in several diseases (see SERCA pumps examples), *Plasmodium* membrane proteins have been studied as potential drug targets. Some examples of membrane proteins targeted in other diseases are cited below.

- Pumps: the Na^+/K^+ ATPase is the target of cardiac glycosides (*e.g.*: ouabain) that are used to treat heart failure and cardiac arrhythmia; the H^+/K^+ ATPases are targeted by benzimidazoles (*e.g.*: omeprazole) in the treatment of reflux and gastric ulcers (Olbe et al., 2003). Bafilomycin (an antibiotic) inhibits V-ATPases.

[15] Tubovesicular network – plays a role in protein transport and contains several parasite and host proteins many of them localized in lipid rafts (Haldar et al., 1989)

- Major facilitator superfamily (MFS): Several inhibitors of the mammalian sodium/glucose co-transporter SGLT2 (Na^+ dependent glucose transporters) are in phase III of clinical trials for the treatment of type II diabetes (Bakris et al., 2009; Staines et al., 2010). The *P. falciparum* hexose transporter (PfHT) is responsible for glucose uptake. It has been shown to be inhibited by D-glucose derivative (compound 3361) in *X. laevis* oocytes (Becker and Kirk, 2004; Joet et al., 2003). This hexose derivative inhibits sugar uptake by the parasite leading to rapid lowering of ATP and loss of pH gradient and other essential functions (Becker and Kirk, 2004). Compound 3361 is also active against resistant strains and kills other related parasites, such as *P. vivax*. PfHT is the only malarial transporter that has been validated both genetically and chemically, confirming the essential role of hexose transporters in asexual parasite stages (Blume et al., 2011; Slavic et al., 2010), besides the fact that there are no paralogues in humans and no identified polymorphism. A codon-optimized form of PfHT1 has been expressed in a recombinant way in yeast enabling high-throughput screening of inhibitors (Blume et al., 2011). Folates metabolism is also essential for *Plasmodium* DNA synthesis and two putative folate transporters have been identified in *P. falciparum* (Martin et al., 2005). In this context it is interesting to note that intermediates in folate metabolism (dihydrofolate reductase-thymidylate synthase – DHFR-TS) are targeted by antimalarial (Sulfadoxine/Pyrimethamine) (see Staines et al., 2010 for review).

- Channels: are responsible for diffusive movement of small molecules across biological membranes, involved in osmotic and oxidative stress regulation, glycerol uptake and disposal of metabolic waste (Pavlovic-Djuranovic et al., 2006). Aquaporines are a family of water/solute channels. PfAQP is the only *P. falciparum* membrane transporter characterized by X-ray crystallography to date (Newby et al., 2008; Staines et al., 2010). However, intraerytrocytic stages of *P. berghei* survive with a KO *pfaqp*, the only phenotype observed for KO parasites is less proliferation. This aquaporine seems to be non-essential for erythrocytic stages of the parasite and the absence of specific inhibitors makes this channel less attractive as an antimalarial target (Kun and de Carvalho, 2009; Staines et al., 2010).

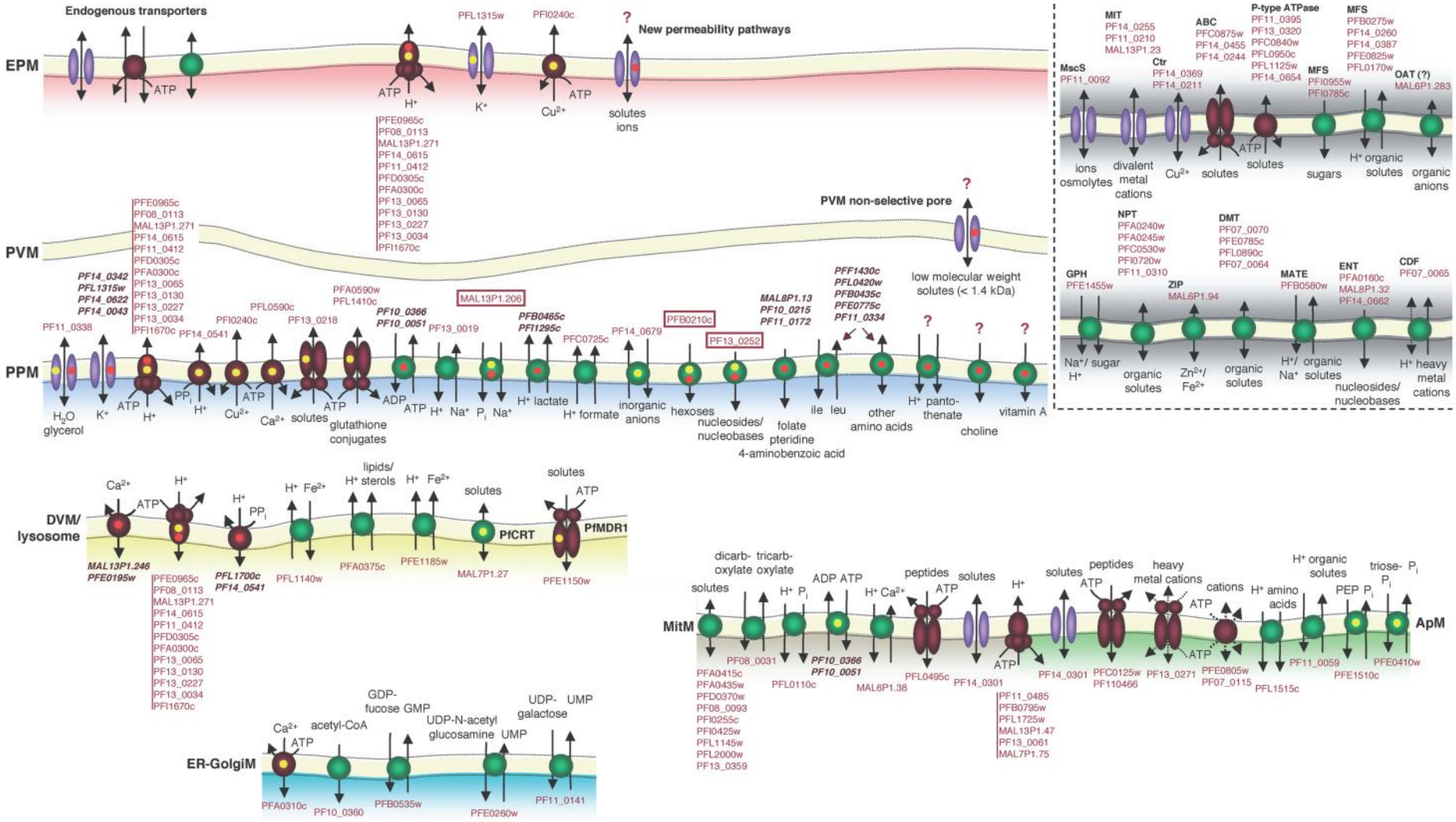
Endogenous transporters
EPM
ATP
ATP
H+
PFL1315w
K+
PFI0240c
Cu2+
ATP
?
New permeability pathways
solutes
ions
PFE0965c
PF08_0113
MAL13P1.271
PF14_0615
PF11_0412
PFD0305c
PFA0300c
PF13_0065
PF13_0130
PF13_0227
PF13_0034
PFI1670c
PVM
PVM non-selective pore
low molecular weight solutes (< 1.4 kDa)
PF14_0342
PFL1315w
PF14_0622
PF14_0043
PF11_0338
PPM
H2O glycerol
K+
ATP
H+
PPi
H+
PF14_0541
PFI0240c
PFL0590c
Cu2+
ATP
Ca2+
PF13_0218
solutes
ATP
glutathione conjugates
PFA0590w
PFL1410c
ADP
ATP
PF10_0366
PF10_0051
PF13_0019
MAL13P1.206
H+
Na+
Pi
Na+
PFB0465c
PF11295c
H+ lactate
PFC0725c
H+ formate
PF14_0679
inorganic anions
PFB0210c
hexoses
PF13_0252
nucleosides/ nucleobases
MAL8P1.13
PF10_0215
PF11_0172
folate pteridine
4-aminobenzoic acid
PFF1430c
PFL0420w
PFB0435c
PFE0775c
PF11_0334
ile
leu
other amino acids
?
?
?
H+ panto-thenate
choline
vitamin A
DVM/ lysosome
Ca2+
ATP
H+
H+
PPi
H+ Fe2+
lipids/ H+ sterols
H+ Fe2+
solutes
solutes
ATP
PfCRT
PfMDR1
MAL13P1.246
PFE0195w
PFE0965c
PF08_0113
MAL13P1.271
PF14_0615
PF11_0412
PFD0305c
PFA0300c
PF13_0065
PF13_0130
PF13_0227
PF13_0034
PFI1670c
PFL1700c
PF14_0541
PFL1140w
PFA0375c
PFE1185w
MAL7P1.27
PFE1150w
ER-GolgiM
Ca2+
ATP
acetyl-CoA
GDP-fucose
GMP
UDP-N-acetyl glucosamine
UMP
UDP-galactose
UMP
PFA0310c
PF10_0360
PFB0535w
PFE0260w
PF11_0141
MitM
solutes
dicarb-oxylate
tricarb-oxylate
H+
Pi
ADP ATP
H+ Ca2+
peptides
ATP
solutes
H+
solutes
peptides
ATP
heavy metal cations
ATP
cations
H+ amino acids
H+ organic solutes
PEP
Pi
triose-Pi
Pi
ApM
PF08_0031
PFA0415c
PFA0435w
PFD0370w
PF08_0093
PFI0255c
PFI0425w
PFL1145w
PFL2000w
PF13_0359
PFL0110c
PF10_0366
PF10_0051
MAL6P1.38
PFL0495c
PF14_0301
PF11_0485
PFB0795w
PFL1725w
MAL13P1.47
PF13_0061
MAL7P1.75
PF14_0301
PFC0125w
PF110466
PF13_0271
PFE0805w
PF07_0115
PFL1515c
PF11_0059
PFE1510c
PFE0410w
MIT
PF14_0255
PF11_0210
MAL13P1.23
ABC
PFC0875w
PF14_0455
PF14_0244
P-type ATPase
PF11_0395
PF13_0320
PFC0840w
PFL0950c
PFL1125w
PF14_0654
MFS
PFB0275w
PF14_0260
PF14_0387
PFE0825w
PFL0170w
MscS
PF11_0092
Ctr
PF14_0369
PF14_0211
MFS
PFI0955w
PFI0785c
OAT (?)
MAL6P1.283
ions
osmolytes
divalent metal cations
Cu2+
solutes
ATP
solutes
sugars
H+ organic solutes
organic anions
NPT
PFA0240w
PFA0245w
PFC0530w
PFI0720w
PF11_0310
DMT
PF07_0070
PFE0785c
PFL0890c
PF07_0064
GPH
PFE1455w
ZIP
MAL6P1.94
MATE
PFB0580w
ENT
PFA0160c
MAL8P1.32
PF14_0662
CDF
PF07_0065
Na+/ H+
sugar
organic solutes
Zn2+/ Fe2+
organic solutes
H+/ Na+
organic solutes
nucleosides/ nucleobases
H+ heavy metal cations

Figure 15 - Overview of P. falciparum identified membrane transporters and their subcellular localization.

Pumps are shown in red, transporters are in green, and channels are in purple. Proteins for which subcellular localization has been directly demonstrated are shown with a yellow dot (the other localizations are just predictions). Proteins marked with a red dot have confirmed transport function, on a physiological and biochemical basis. Candidate genes are annotated in dark red, bold, italics; and the lack of known gene is marked with question mark (?). Gene accession code is put in a box when the specific substrate has been demonstrated. Red vertical lines indicate multi-subunit transport complex. Proteins not marked with neither a yellow nor a red dot, the substrate specificity and the sub-cellular localization are predicted by protein conserved domains or homology with known transporters of other organisms. When in dashed line the subcellular localization could not been predicted. Abbreviations are as follows: ***ABC****- ATP-binding cassette superfamily;* ***ApM****- apicoplast membrane;* ***CaCA****- Ca $^{2+}$-cation antiporter family;* ***CDF****- cation diffusion facilitator family;* ***Ctr****- copper transporter family;* ***DMT****- drug/metabolite transporter superfamily;* ***DVM*** *– digestive vacuole membrane;* ***ENT****- equilibrative nucleoside transporter family;* ***EPM****- erythrocyte plasma membrane;* ***ER-GolgiM****- endoplasmic reticulum and Golgi membrane;* ***GPH****- glycoside-pentoside-hexuronide-cation symporter family;* ***MATE****- multi-antimicrobial extrusion family;* ***MscS****- small conductance mechanosensitive ion channel family;* ***MFS****- major facilitator superfamily;* ***MIT****- CorA metal ion transporter family;* ***MitM****- mitochondrion membrane;* ***NPT****- novel putative transporter family;* ***OAT****- organo anion transporter family;* ***PEP****- phosphoenolpyruvate;* ***PfCRT****- chloroquine resistance transporter;* ***Pgh1****- P-glycoprotein homologue 1;* ***PPM****- parasite plasma membrane;* ***PVM****- parasitophorous vacuolar membrane;* ***ZIP****- zinc (Zn^{2+})-iron (Fe^{2+}) permease family. For the purpose of this figure the four apicoplast membranes are represented as a single membrane; likewise the two mitochondrial membranes are represented as a single membrane.* (Van Dooren et al., 2000; Gardner et al., 2002; Martin et al., 2005, 2009).

- ATP binding cassette: PfMRP (*P. falciparum* multi drug resistance protein) (Raj et al., 2009; Staines et al., 2010) and the Pgh1 the P-glycoprotein homologue 1, encoded by *pfmdr1* gene (Cowman et al., 1991; Fidock et al., 2000; Martin et al., 2009), are both localized at the parasite's digestive vacuole membrane (Figure 15), and have been suggested to play a key role in the resistance to antimalarials (Fidock et al., 2000; Jiang et al., 2008; Martin et al., 2009). These resistance mechanisms are not fully understood but increased gene copy number and mutations may be related. PfCRT when mutated confer a chloroquine resistance phenotype, and mutations in Pgh1 enhance this phenotype in resistant parasites. Chloroquine resistance phenotype is observed by the reduction of the accumulation of chloroquine in the digestive vacuole (Becker and Kirk, 2004; Martin and Kirk, 2004). Inhibiting Pgh1 can revert the resistance phenotype. Both transporters have been expressed in heterologous systems (Martin et al., 2009).

- Nucleoside transporters: required for nucleotide (ATP) and nucleic acids production. The human hENT1 (Equilibrated Nucleoside transporter 1) is the target of vasodilatory drugs (Dilazep and dipyridamole). PfENT1 is responsible for external supply of purine nucleotides, essential for parasite survival (transgenic parasites lacking PfENT1 do not survive without purine supplement at supra-physiological concentrations). TbAT1 is an adenosine/adenine transporter, target of melarsoprol for sleeping sickness treatment (*Trypanosoma brucei*).

- Amino-acid transporters and other secondary transporters: *Plasmodium* can meet most of amino acid requirements by host haemoglobin digestion except for isoleucine, this may be a pathway worth exploring for new antimalarial research (Liu et al., 2006). PfCRT, the *Plasmodium falciparum* chloroquine resistance transporter, is implicated in chloroquine resistance and is localized on the food vacuole membrane (Martin and Kirk, 2004; Martin et al., 2009).

- Cation exchangers: the Ca^{2+}/H^{+} exchanger (PfCAX) is a good potential antimalarial target as it has no human orthologue (McAinsh and Pittman, 2009). PfNHE is a Na^{+}/H^{+} exchanger, essential for the regulation of intracellular pH and extracellular Na^{+} concentration (Bennett et al., 2007; Ferdig et al., 2004). It has been linked to quinine resistance and inhibited by the diuretic drug amiloride (Jiang et al., 2008). It is also inhibited by cariporide, a drug also used to reduce the risks in patients undergoing coronary artery bypass surgery (see Staines et al., 2010 for review). However, it was proven to be non-essential for the parasite (Guttery et al., 2013).

- Apicoplast proteins: are very promising targets, as most of them do not possess human orthologs (prokaryotic origin). PfiTPT and PfoTPT, transport the Pi that has been previously transferred to sugars. These proteins are plant like transporters and possess no human orthologues (see Staines et al., 2010 for review).

- Mitochondrial pathways: are implicated in electron transfer chain (ETC) known to be malign to parasites when disrupted as it participates in ROS liberation), citric acid cycle, ubiquitine biosynthesis. One of the advantages in targeting mitochondrial pathways is that there is only one mitochondria in asexual stages of *P. falciparum.* PfAdT (the mitochondrial ATP/ADP translocase) is considered one of the most promising antimalarial targets (Staines et al., 2010). Protein from the ETC are also very promising antimalarial targets and are already targeted by some available antimalarials (eg., atovaquone and quinolones that both target the *bc1* complex) (Fry and Pudney, 1992; Nixon et al., 2013a, 2013b).

II.3 – Heterologous Expression Systems for Plasmodium Transporters

To obtain transporters of *P. falciparum* one cannot simply isolate them directly from the parasite, as they express in insufficient amount. It is then recommended to express them in heterologous systems. Some of these systems used are described below.

Escherichia coli is still one of the preferential hosts for heterologous protein expression due to its ease in genetic manipulation, low cost and rapid culture. Nevertheless, it is not the optimal host for eukaryote protein expression due to inappropriate post-translation modifications, expression in inclusion bodies and toxicity of some *Plasmodium* genes and proteins (reviewed by Birkholtz et al., 2008). Several large scale attempts of producing *P. falciparum* proteins in *E. coli* have been attempted (Mehlin et al., 2006; Vedadi et al., 2007). A study selected 400 *distinct P. falciparum* and related Apicomplexa genes ending in 36 crystal structures (Vedadi et al., 2007). Many other soluble proteins and vaccines targets have been expressed in *E. coli* using pET vectors and the BL21 (DE3) strain.

Toxoplasma gondii is also a pathogen parasite from the Apicomplexa phylum (as *Plasmodium*). It is easier to genetically manipulate, hence it is possible to perform functional studies or create mutants (reviewed by Birkholtz et al., 2008).

The single cell amoeba *Dictyostelium discoideum* is gaining popularity for *Plasmodium* protein expression, especially transmembrane transporters. It is easy to maintain in culture, it possesses the classical eukaryotic intracellular organels and cell biology features, genes may be disrupted by homologous recombination or silencing, and presents an AT codon bias similar to *Plasmodium's* (reviewed by Birkholtz et al., 2008). PfCRT was expressed in the digestive compartments of *D. discoideum*, giving functional evidences for the role of mutations in this protein in reducing chloroquine accumulation and in chloroquine resistance (Naudé et al., 2005).

Yeast (*Pichia pastoris* and *Saccharomyces cerevisiae*) are a good alternative to *E. coli*, rapid and low-cost eukaryote option for heterologous expression of *P. falciparum* proteins (*e.g.* Cardi et al 2010a). *S. cerevisiae* is a good option because of the knowledge of its molecular biology and physiology. It presents appropriate post-translational modifications (e.g., disulphide bond and protein folding), and avoids precipitation of proteins into inclusion bodies. However, *S. cerevisiae* recognizes AT rich segments as termination signals, hence genes with optimized codons for expression in this organism are often needed, removal of poly (A) tails and 5' - Kozak sequences can also improve expression. It is a great tool for complementation studies as a large variety of strains are available (reviewed by Birkholtz et al 2008). This system has allowed functional studies of *P. falciparum* transmembrane proteins such as the vacuolar H^+ - ATPase (Yatsushiro et al., 2005) and the aquaglyceroporin (Zeuthen et al., 2006). Moreover it allowed expression of recombinant transmission-blocking vaccines (Kaslow et al., 1994; Moelans et al., 1995; Stowers and Carter, 2001).

Baculovirus-infected insect cells can also be used. The gene is cloned into a vector that, by homologous recombination, will integrate the viral genome. The virus will use the eukaryotic host insect cell (*Spodoptera frugiperda*) machinery to transcribe the gene and express the final protein. The great advantage of this technique, besides eukaryote post-translational modifications, is that the folding and assembly of the newly formed protein is similar to what happens in *Plasmodium*

(reviewed by (Birkholtz et al., 2008). This system enabled the expression of vaccine targets such as: recombinant domains of merozoite surface protein MSP-1 (Chang et al., 1992; Pizarro et al., 2003), CSP (Jacobs et al., 1991; Murphy et al., 1990) and a erythrocyte membrane protein PfEMP1 variant (Barfod et al., 2007). These expressed proteins retained their native conformational epitopes and triggered immune responses.

Plants have also been used to express *Plasmodium* proteins. Plants are easily cultured in bioreactors and have the advantage of well folding proteins and appropriate post-translational modifications. They have been used for vaccine production as for instance, in the production of MSP1 protein in tobacco plant cells (Ghosh et al., 2002).

Expression in *Xenopus laevis* has been previously reported to be one of the best systems for functional analysis (Martin and Kirk, 2004). One of the major advantages is that it has a preferential codon usage similar to *Plasmodium's.* This organism is difficult to manually manipulate, cDNA injection is labor-demanding, it displays a rather heterogeneous expression between batches, and it is difficult to obtain a sufficient amount of material. It is useful for functional studies of transmembrane transporters (reviewed by Birkholtz et al., 2008). Several *P. falciparum* membrane transporters described as expressed in *X. laevis* oocytes such as: *P. falciparum* hexose transporter PfHT1, enabling the definion of its substrate specificity (Woodrow et al., 1999); nucleoside transporter PfENT1 (Carter et al., 2000; Downie et al., 2008, 2006; Parker et al., 2000), identifying the transported substrate and further studies as a potential antimalarial target; the Na^{+}-phosphate transporter PfPiT (Saliba et al., 2006), and the aquaglyceroporins PfAQP (Hansen et al., 2002), but also PfCRT, leading to the proposal of a role for chloroquine resistance (Martin et al., 2009; Nessler et al., 2004) and Pgh1 (Sanchez et al., 2008).

Mammalian cells are well characterized, with versatile protocols and genetic manipulations, as well as appropriate post-translational modifications and correct folding of proteins. However, the expression yields are low. These cultures are more expensive, and more difficult to grow, and they also require a codon bias optimization. They can be used for complementation assays and functional characterization. Some widely used mammalian cells are COS cells (fibroblast-like cell line originally from monkey kidney), HEK293 cells (Human Embryonic Kidney 293 cells), and CHO cells (Chinese hamster ovary cells) (See (Martin et al., 2009) for review). This system was very usefull in expressing *Plasmodium* proteins involved in host-parasite interactions, enlightening mechanisms like erythrocyte invasion and parasite sequestration to the placenta and vascular endothelium.

Cell-free expression systems are an advantage for proteins that undergo proteolysis or that usually accumulate in inclusion bodies. They present high expression yields without the associated toxicity when expression is performed in cell systems (reviewed by (Birkholtz et al., 2008). Some *P. falciparum* soluble proteins were expressed in this system: the antimalarial target PfDHFR-TS (Mudeppa et al., 2007); and several vaccine targets (Pfs25, PfCSP and PfAMA-1) using both native and codon-optimized genes (Tsuboi et al., 2008); though it remains challenging for the expression of transmembrane proteins.

II.4 – Generalities on P- ATPases

Membrane ATPases are a class of transmembrane proteins that transport with high affinity inorganic ions and organic solutes, the necessary energy being provided by ATP hydrolysis. There exist three functional classes of ionic ATPases: i) F- type ATPases, V- type ATPase and P- type ATPases; and one non ionic transporter: ATP Binding Cassette transporters (Møller et al., 2010; Pedersen, 2007; Shechter and Rossignol, 1997).

P-type ATPases are membrane proteins of 70 – 150 kDa, generally with an even number of transmembrane domains, the carboxyl and amino termini are then both localized on the cytoplasmic fraction. They are present in eukaryote and prokaryote plasma and intracellular membranes, with a characteristic pattern of conserved amino acids D/NKTGTLT (Kühlbrandt, 2004; Møller et al., 1996). During their activity cycle a phosphorylated intermediate protein is formed, from which they inherited the name of P-ATPases (Møller et al., 2010; Shechter and Rossignol, 1997). Some examples of ATPases included in this category are:

1. Na^+/K^+ ATPase responsible for maintaining the sodium and potassium gradient in the plasma membrane of animal cells and also the transmembrane electric potential. These ATPases are inhibited by ouabain, a cardiac glycoside (Skou, 1998).

2. H^+ ATPases are present on the plasma membrane of vegetal and yeast cells. They create an electrochemical gradient that is used by several secondary active transporters.

3. H^+/K^+ ATPase are mainly localized on the plasma membrane of gastric cells. They hydrolyse ATP and exchange H^+ with K^+ from the extracellular space. They are able to maintain the cyotosol pH at around 7, while the extracellular pH is at around 1.

4. In this class are also included ATPases responsible for the transport of so called “heavy” metals (*e.g.*, Cu^{2+}, Zn^{2+}, Cd^{2+}).

5. Ca^{2+} ATPases transport calcium ions through membranes by ATP hydrolysis: The Plasma Membrane Calcium ATPases (PMCA), and the Sarcoplasmic/Endoplasmic Calcium ATPases (SERCA) (Figure 16), are responsible for taking calcium out of the cell cytoplasm (Clapham, 2007).

P- ATPases can be divided in 5 evolutionary groups, subdivided in 10 subclasses that are specific for a substrate ion. (Figure 15)

- Type-I ATPases are possibly the most ancient group and hence the most simple.
    - Type IA – bacterial ion pumps
    - Type IB – mostly found in bacteria, transport soft transition metal ions (Cu^{2+}, Ag^+, Zn^+, Pb^{2+}, Cd^{2+})

- Type-II and III – are responsible for maintaining the membrane potential
    - Type II is the most diverse group. Type-IIA includes SERCA transporters (see Figure 15); Type IIB, PMCA proteins (see also Figure 16). Type IIC includes Na^+/K^+ ATPases and H^+/K^+ ATPases. And Type-IID – ENA transporters.
    - Type III A are H^+-pumps principally localized on the plasma membrane of plants and fungi, responsible for maintaining pH gradient.
- Type-IV and V – were only found in eukaryotes, until date. The natural substrate of Type V ATPases is presently unknown. Type IV ATPases most probably transport lipids and are responsible for the maintenance of lipid bilayer asymmetry (flippases).

P-ATPases are usually composed of: a cytosolic domain responsible for ATP hydrolysis (by phosphorylation of an aspartate residue); a membrane domain constituted of transmembrane helices; and a small luminal domain. Vanadate and curcumin (Bilmen et al., 2001) inhibit P-type ATPases by interaction with the phosphorylation and nucleotide-binding domain, preventing the activation of the enzyme, while thapsigargin and derivatives or CPA inhibit transport by binding in the membrane pocket (Laursen et al., 2009). During its cycle, these enzymes pass by two different conformational states, which depended on where the ion binding pocket is oriented: E1 (orientation towards exterior, high ion affinity state) and E2 (orientation towards interior, low ion affinity state) (see Figure 17, for the SERCA Ca^{2+}-ATPase example). These transitional conformations enable the transport of the ion from one side of the membrane to the other (Kühlbrandt, 2004; Møller et al., 2010).

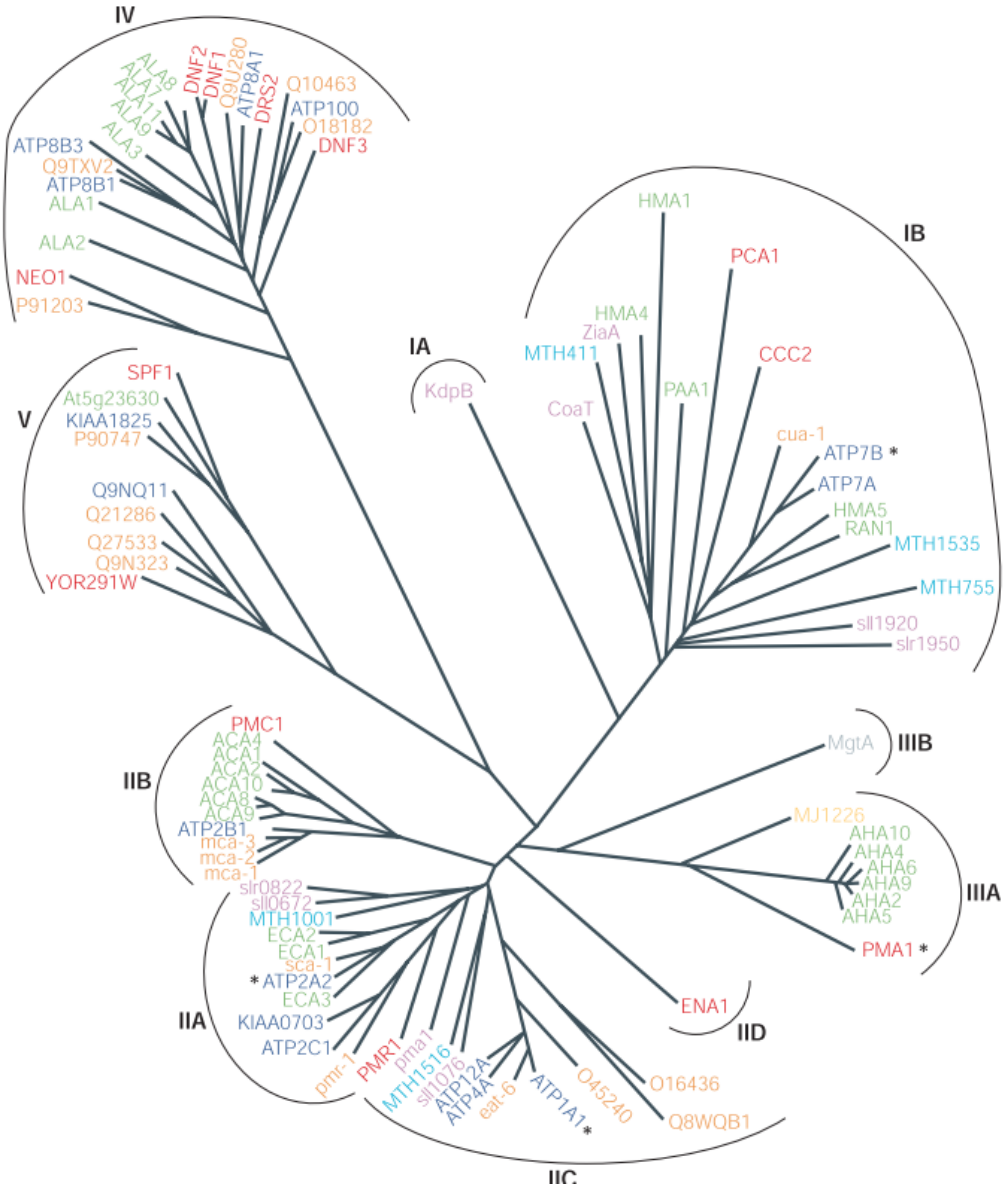

Figure 15 - Phylogeny of P-type ATPase family.
(Kühlbrandt, 2004)**.**

II.5 - Generalities on Calcium ATPases

Calcium is the most important and versatile secondary messenger. Calcium does not directly transduce signal, but it is transmited by proteins that bind to Ca^{2+} and then trigger the signalization pathway. Cells use its binding energy for signal transduction and also to trigger protein conformation changes and alter local electrostatic fields (Clapham, 2007). Calcium is implicated in numerous cellular processes such as neurotransmission, immunity, cell signalization, fertilization, apoptosis,

gene transcription, muscle contraction, egress, cell invasion, intracellular development, motility, secretion, and generation of fuels in several metabolic pathways. Organisms are always avidly trying to take calcium out of the cytosol because it precipitates phosphate groups and its presence in high concentration becomes very toxic. As calcium is so important in cellular processes, the concentration of this ion is crucial and must be tightly regulated (Brini and Carafoli, 2009; Carafoli, 2005, 2002).

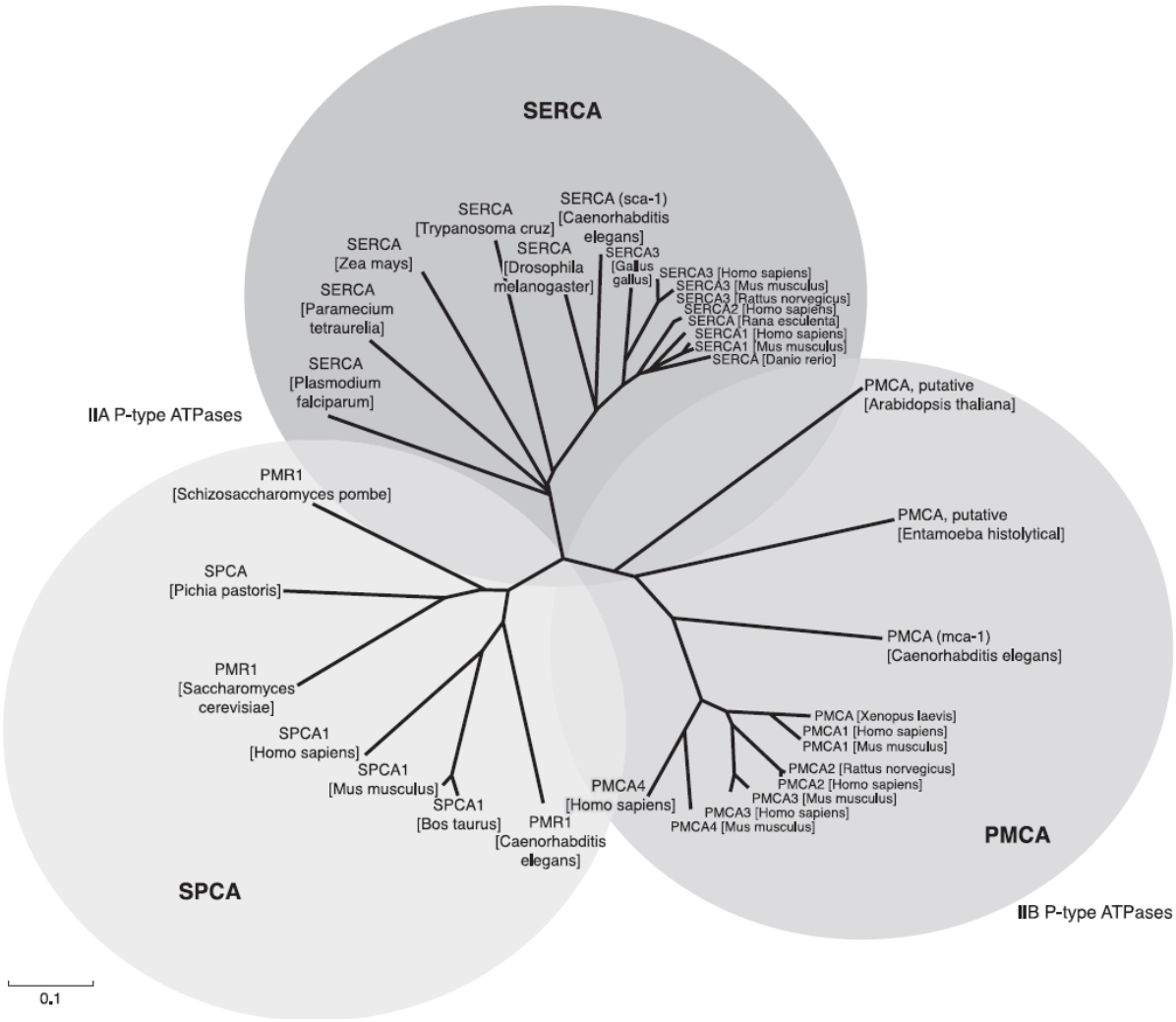

Figure 16 - Phylogenetic representation of calcium-ATPase transporters in higher animals*. sarco-endoplasmic reticulum (SERCA), the Golgi network (SPCA) and the plasma membrane (PMCA) calcium ATPases*
(Brini and Carafoli, 2009).

Three major classes of calcium ATPases pumps have been described in higher animals and are located in the: sarco-endoplasmic reticulum and in extension in the nuclear envelope (SERCA), the Golgi network (SPCA) and the plasma membrane (PMCA). Phylogenetically these calcium ATPases are separated in two subgroups: subgroup IIA that includes SERCA and SPCA pumps; and subgroup IIB that groups PMCA transporters (see Figure 16).

II.6 – SERCA Pumps

II.6.1 – Calcium transport

SERCA is a calcium transporter responsible for pumping the calcium out of the cytosol and store it in the lumen of the sarcoplasmic reticulum, enabling rapid muscle relaxation after contraction has occurred. SERCA constitutes 70% of the sarcoplasmic membrane proteins. It is regulated by phospholamban (a 52 kDa protein) that, when phosphorylated, inhibits SERCA activity; and sarcolipin (Montigny et al 2014, submitted). SERCA are specifically inhibited by thapsigargin, a sesquiterpene lactone produced by the plant *Thapsia garganica*, that blocks the protein in the E2 form; by cyclopiazonic acid (CPA); and by 2,5-di-tert-butylhydroquinone (BHQ).

Calcium transport is achieved by a tightly regulated cycle that drives transformation of the chemical energy of an aspartyl-phosphorylated intermediate into an active transport of cations (Møller et al., 2010). Many studies contributed to the description of the transport, in particular functional studies after site-directed mutagenesis, but also biophysical studies, proteolysis, etc (Møller et al., 2010, 1996). Structural studies in particular conditions enabled elucidation of intermediary states of the calcium ATPase cycle (Jensen et al., 2006; Montigny et al., 2007; Olesen et al., 2007; Picard et al., 2006; Sørensen et al., 2004; Toyoshima et al., 2000). Conformation changes of the Ca^{2+} - ATPase state E2 to E1 (step 1, Figure 17 A) is driven by the binding of two calcium ions (step 2, Figure 17 A). It is followed by the autophosphorylation of aspartate 351 by hydrolysis of a molecule of ATP (step 3, Figure 17 A), resulting in a high energy phosphorylated E1-P-Ca^{2+} intermediate with occluded calcium ions. The ATPase conformation changes to a low energy E2-Ca^{2+} phosphorylated state (step 4, Figure 17 A). Then the two calcium ions are released into the lumen of the endoplasmic reticulum, by opening of the enzyme transmembrane domains (step 5, Figure 17 A). This is accompanied by the exchange of 2-3 protons that are then occluded in inside the membrane, that partially restore electrostatic balance the transmembrane segment (step 6, Figure 17 A). Dephosphorylation of the E2 state of the SERCA (step 7, Figure 17 A) will allow the cycle to undergo again, by changing to an E1 state that will receive the calcium ions by exchange of the protons occluded (Figure 17 A) (Brini and Carafoli, 2009; Danko et al., 2009a; Møller et al., 2010).

SERCA1a, from rabbit sarcoplasmic reticulum (SR), has been extensively studied due to the ease in preparation directly from rabbit skeletal muscle, especially fast twitch muscles.

The crystallographic 3D structure of this protein was the first P-ATPase solved (Toyoshima and Nomura, 2002; Toyoshima et al., 2000). In 2005 the expression of rabbit Serca1a in yeast and its purification led to the crystallization of this protein (Figure 18) (Jidenko et al., 2005). The purified and relipidated SERCA1a presented the same structure as the native protein. This protocol of heterologous expression and affinity-chromatography purification enabled to retrieve the functional and structural properties characteristic of SR Ca^{2+} - ATPase. Later, the crystallization of some interesting mutants was possible using the established protocol (Clausen et al., 2013; Marchand et al., 2008). The main results from those studies included a number of functional characterization; and the "ping-pong" mechanism of Ca^{2+} transport between the membranous part and the phosphorylation domain was finally described (Figure 19). This mechanism requires a delicate balance in the H^+ binding between transmembrane helices (Clausen et al 2013).

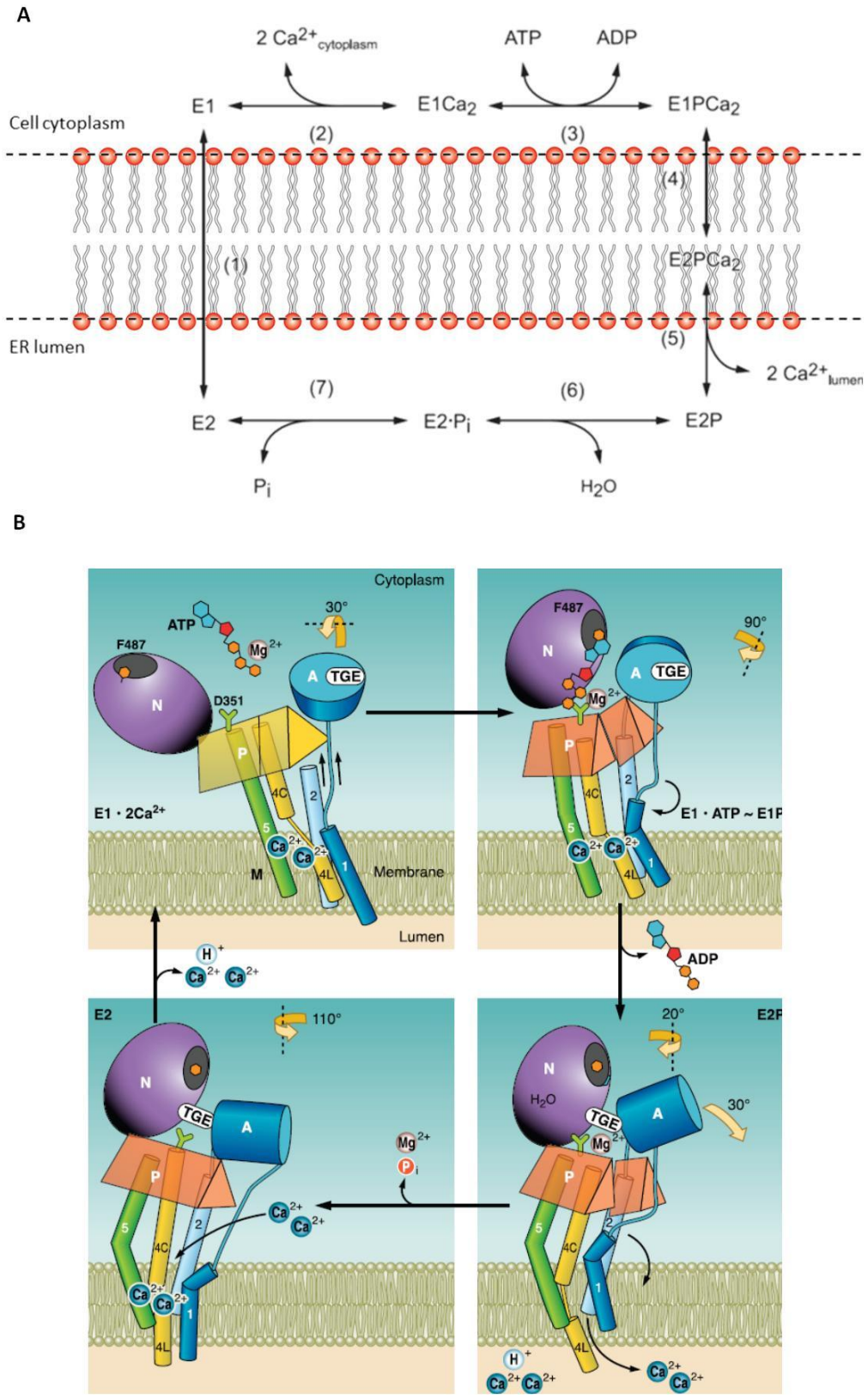

Figure 17 - Schematic representation of the cycle of a SERCA Ca^{2+} - ATPase.

A- *Simplified representation of Ca^{2+} - ATPase cycle;* ***B-*** *Representation of SERCA conformational changes that occur during phosphorylation and calcium transport* (Danko et al., 2009b; Toyoshima, 2008).

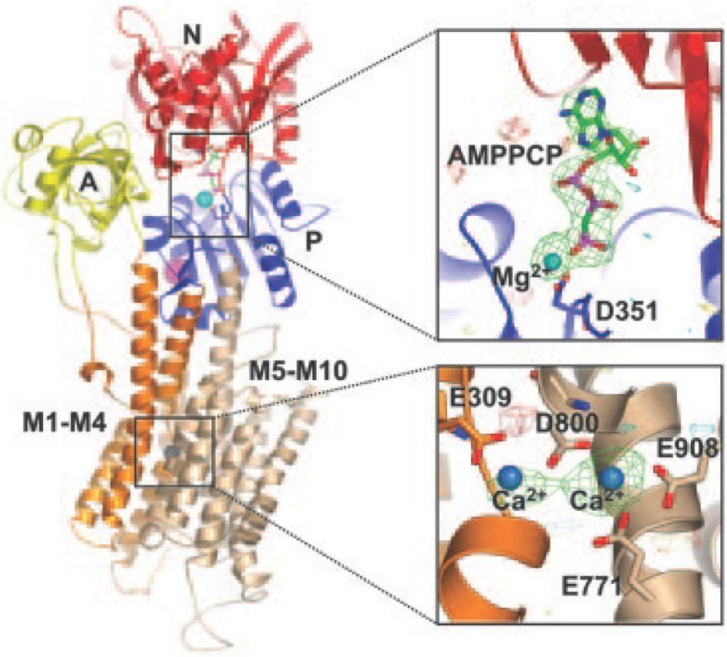

Figure 18 - Native structure of SERCA1a expressed in S. cerevisiae.

Actuator domain (A) is represented in yellow, nucleotide-binding domain (N) in red, and phosphorylation domain (P) in blue. Crystallization was undertaken in presence of AMPPCP, a non-hydrolyzable ATP analog (Jidenko et al., 2005).

The structure of the SERCA Ca^{2+} - ATPase can be divided in four important domains, one membranar domain and three cytosolic domains which are: the nucleotide-binding (N) domain, the phosphorylation (P) domain, and the actuator (A) domain (Figure 17 B and Figure 18). The nucleotide (N) binding domain is strongly linked to the P domain. It is responsible for binding of Mg^{2+}-ATP. The phosphorylation (P) domain is the core of SERCA proteins with the sequence DKTGTLT. It is phosphorylated by ATP hydrolysis, and together with the actuator (A) domain conformational changes occur and translocation takes place (Figure 19 and Figure 17 B). The A domain is also conserved and it is the smallest cytoplasmic domain. The phosphorylation of the SERCA enzyme is achieved by the hydrolysis of the ϒ-phosphate of ATP, which covalenty attaches on an aspartate 351, in a highly conserved sequence of the ATPase: SDKTGT [L/I/V/M] [T/I/S] (Kühlbrandt, 2004; Møller et al., 2010). The membranous (M) domain corresponds to the 10 helical transmembrane domains (M1-M10), embedded in the lipid bilayer, forming an ellipsoidal cylindrical structure. It can be divided in three subdomains: two N-terminal formed by the couples M1 – M2 and M3 – M4, the third one is C-terminal M5-M10 and forms a tight and tangled helical bundle. In the E1 state Ca^{2+} bind at two sites locate between M4, M5 M6 and M8. The ion binding sites are in the M-domain.

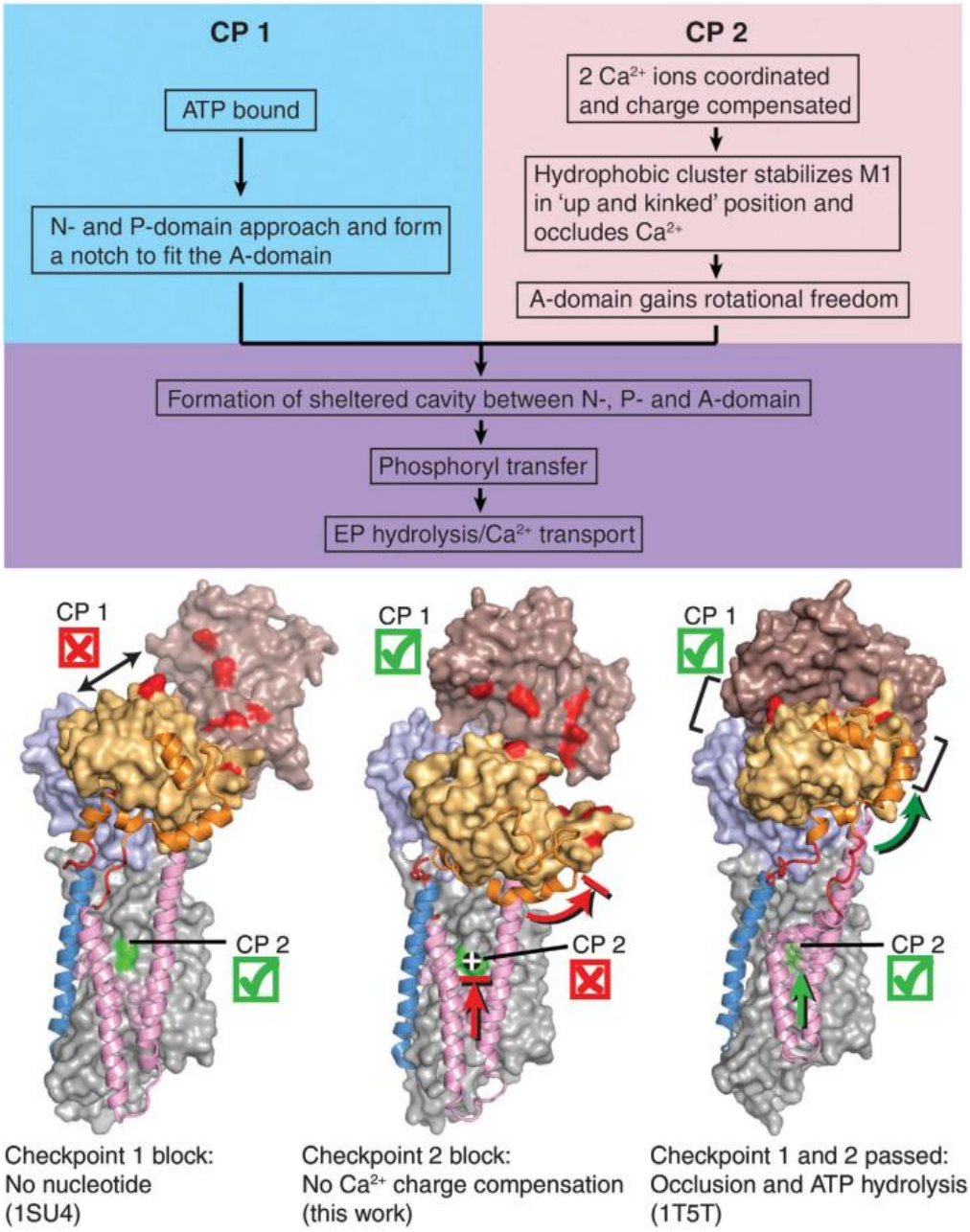

Figure 19 - Schematic representation of the structural requirements of SERCA for Ca^{2+}-dependent activation of phosphorylation by ATP.

The three structures here represented are (from left to right): wild-type Ca_2E1 (Toyoshima et al., 2000)*, E309Q_Ca_2E1 (AMPPCP)* (Clausen et al., 2013)*, and wild-type[Ca_2]E1-AlF_4-ADP* (Sørensen et al., 2004)*. Requirements at both 'checkpoints', CP1 and CP2, have to be met; otherwise, the enzyme activity will be strongly lowered or completely abolished. Pink - M1 and M2; blue - M3; red - A-M1 and A-M3 linker regions; orange - N-terminal part of the A-domain; brown - N-domain; light blue - P-domain; light orange - A-domain; light grey - transmembrane domain. Some of the amino acids involved in interdomain contacts and Ca^{2+} binding are highlighted by red and green colouring, respectively. CP – check-point. Source:* (Clausen et al., 2013).

II.6.2 – SERCA pumps and disease

SERCA pumps are involved in several diseases. Two human genetic diseases related to mutations in SERCA pumps have been defined: Brody's disease (rare recessive muscular condition characterized by impaired relaxation, muscle cramps and stiffness following exercise) and Darier's disease (rare autosomal dominant skin disease defined by loss of adhesion between epidermal cells and abnormal keratinization, keratotic papules in seborrhoic areas and skin flexures). Moreover, decreased expression or mutations in *SERCA* genes lead to cardiac diseases such as heart failure and cardiomyopathy (Kranias and Bers, 2007). Such mutations are also involved in type II diabetes (Varadi et al., 1999) and cancer (Monteith et al., 2007). In cancer, other mechanisms might be involved such as SERCA dysfunction that leads to deregulated calcium homeostasis triggering abnormal cell proliferation (Brini and Carafoli, 2009; Monteith et al., 2007).

Thapsigargin has been proposed as a treatment for prostate cancer, administrated as an inactive prodrug that would be activated in some cancerous cells. In fact, prostate cancer cells are known to produce a serine-threonine protease that is used to activate this prodrug (Denmeade et al., 2012). Curcumin, a SERCA inhibitor with low toxicity, is also actually in phase II of clinical trials as a treatment for cancer (Anand et al., 2008; Dhillon et al., 2008). These are some evidences that SERCA proteins can be specifically drug-targeted in certain diseases.

II.7 - PfATP6 and Calcium signaling in Plasmodium parasites

II.7.1– Calcium signaling in *Plasmodium*

Calcium has been shown to be essential at several stages of parasite development but its regulation is badly understood. After invasion, an important increase in Ca^{2+} concentration occurs in the parasitiphorous vacuole (Luo et al., 2005; Plattner et al., 2012). Blocking calcium influx inhibits parasite growth, arresting the development at the ring stage (Krogstad et al., 1991). The food vacuole and the endoplasmic reticulum (ER) are internal Ca^{2+} stores (Plattner et al., 2012), and the calcium homeostasis is ensured by: SERCA; the plasma membrane ATPase related transporters (PTM); three ATPases located in the Golgi-endoplasmic reticulum; and a single Ca^{2+}/H^{+} exchanger (PfCAX) (Nagamune and Sibley, 2006).

II.7.2– PfATP6

PfATP6 is the only SERCA of *Plasmodium falciparum* (Kimura et al., 1993). It is localized on the endoplasmic reticulum of the parasite that is responsible for the transport of Ca^{2+} ions from the cytosol to the endoplasmic reticulum. *Pfatp6* gene is 4.3 kb long (3684 bp in the coding region) and has 3 exons and 2 introns and is localized on *P. falciparum* chromosome 1. It is the SERCA type gene that possesses the largest sequence deposited in NCBI (Dahlström et al., 2008; Jambou et al., 2010; Tanabe et al., 2004).

PfATP6 protein has a molecular mass of 139.4 kDa, with 10 putative transmembrane domains and the characteristic cytosolic catalytic domains of a SERCA protein. It presents a phosphorylation site, a nucleotide binding N-domain, and two Ca^{2+} binding sites. Sequence analysis reveals a 39% of identity between SERCA1a and PfATP6 that is increased to 53% when the N-domain is excluded (Figure 20) (Arnou et al., 2011; Cardi et al., 2010b). All of these characteristics include PfATP6 in the P-type ATPase class along with SERCA proteins, in the IIA subgroup for Ca^{2+} transport (Kimura et al., 1993) (Figure 15). The major differences between PfATP6 and all other SERCAs are mainly localized in the N domain, facing the cytoplasmic side of the endoplasmic reticulum (Dahlström et al., 2008; Jambou et al., 2010; Tanabe et al., 2004) (Figure 20). This domain has 200 additional residues than SERCA1a and an abnormal quantity of poly-asparagines motifs, compared to other eukaryotes and these are predicted to form non-globular structures (Aravind et al., 2003; Arnou et al., 2011) (Figure 20). These motifs could be implicated in the evasion from the host immune system (Anders, 1986), but their exact role is still under debate. PfATP6 is inhibited by SERCA classical inhibitors such as thapsigargin, CPA and BHQ. CPA has the best affinity for PfATP6 ~0.4 µM, then comes BHQ with ~65 µM, and finally thapsigargin with more than 150 µM (IC_{50} values) (Arnou et al., 2011). CPA inhibition is better for PfATP6 than for SERCA1a, the inverse is observed with thapsigargin. Thapsigargin and CPA sensitivity and binding sites were proven to be different in the mammalian and the *P. falciparum* ortholog (Kotšubei et al., 2013; Plattner et al., 2012).

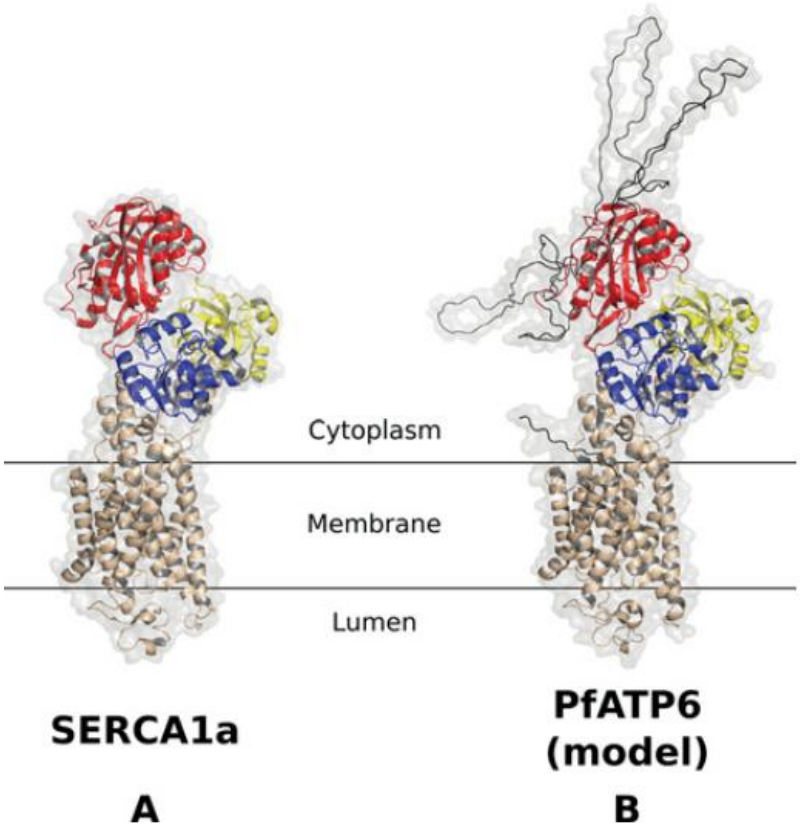

Figure 20 - Comparison of SERCA1a crystallographic structure to a structural model of PfATP6.

*The structure of **A)** SERCA1a in E2 $MgFl_2$ complex with bound CPA and AMPPCP*; *and **B)** the homology model of PfATP6 in the same state. A-(actuator) domain is yellow, the N-domain is red, the P-(phoshorylation) domain is blue and the membrane domain is wheat* (Arnou et al., 2011).

II.7.3– PfATP6 as the proposed target of artemisinins

Since 2003, PfATP6 gained a great interested from the scientific community when it was described to be the direct target of a potent antimalarial: artemisinin (Eckstein-Ludwig et al., 2003).

This proposition was based on the structural similarities between thapsigargin and artemisinin. Thapsigargin is a potent inhibitor of mammalian SERCA, and has a somewhat similar structure to artemisinin, as it is also a sesquiterpene lactone; however lacking a peroxide bond (Figure 21). This peroxide is crucial for artemisinin efficiency. Other molecular dynamic studies suggested that the introduction of an endoperoxide bridge in thapsigargin (thaperoxide 22) increased 100 fold the effect on PfATP6, based on a homology model of this transporter (Helal and Avery, 2012). Later docking simulations on a model of PfATP6 suggested that artemisinin, could bind to PfATP6 through hydrophobic interactions (Jung et al., 2005; Naik et al., 2011). Other *in silico* studies using amino acid similarities in conserved domains between P-type ATPases, SERCA, and PfATP6, and the prediction of PfATP6 binding pocket when coupled with thapsigargin, showed that Fe^{2+}-artemisinin binds preferentially to PfATP6, (Jung et al., 2005; Shandilya et al., 2013; Naik et al., 2011). Except that other publications also based on docking simulations, found opposite results (Garah et al., 2009).

Lepore and collaborators recently performed modeling experiments for several SERCA proteins (*P. falciparum, Homo sapiens, Schistosoma mansoni*) and docking of artemether. No significant differences in the mode of binding of artemether to these different SERCA was found (Lepore et al., 2011). SmSERCA possesses a natural 263E residue and *S. mansoni* is still sensitive to artemisinin (Cui et al., 2012). L263E mutation was introduced in *P. falciparum* 3D7 through allele exchange and did not show any differences in artemisinin derivatives sensitivity, although the authors affirm a tendency towards reduced susceptibility (Stephanie Gaw Valderramos et al., 2010).

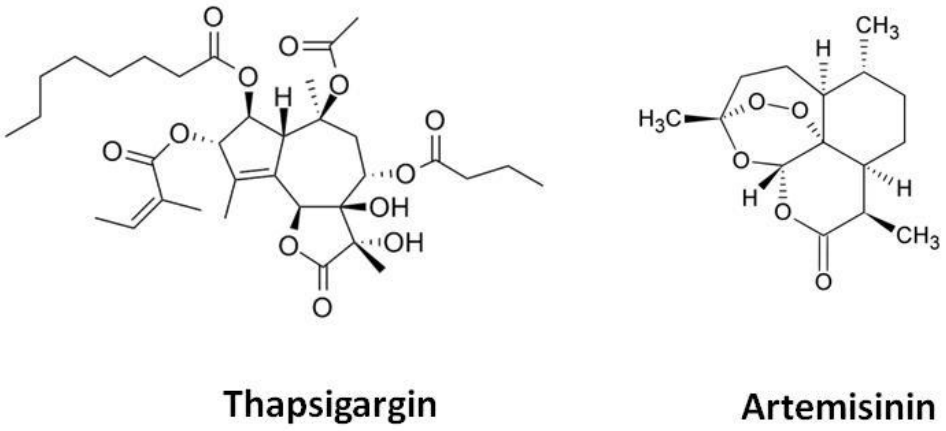

Figure 21 - Comparison of thapsigargin and artemisinin chemical structures.

Krishna and collaborators reported the expression of PfATP6 in *Xenopus laevis* oocytes membranes (1% of total membrane proteins), to overcome the impossibility to performe a functional assay in COS cells (Eckstein-Ludwig et al 2003). The oocytes membranes expressing PfATP6 apparently

showed a higher Ca^{2+}-dependent ATPase activity. They reported that PfATP6 was inhibited by thapsigargin (Ki ~64 nM), CPA (Ki < 1µM) and vanadate (IC_{50} ~100 µM, general inhibitor of P-type ATPases) but not by ouabain (Na^+/K^+ ATPase inhibitor) (Figure 22 A). Artemisinin at 1 µM (Ki of 150 nM) was reported to inhibit ATPase activity in membranes expressing PfATP6, but not chloroquine nor quinine (Figure 22 B). Although this value was higher than the IC_{50} on parasites, they claimed that values for targets are generally higher than what is observed on parasites.

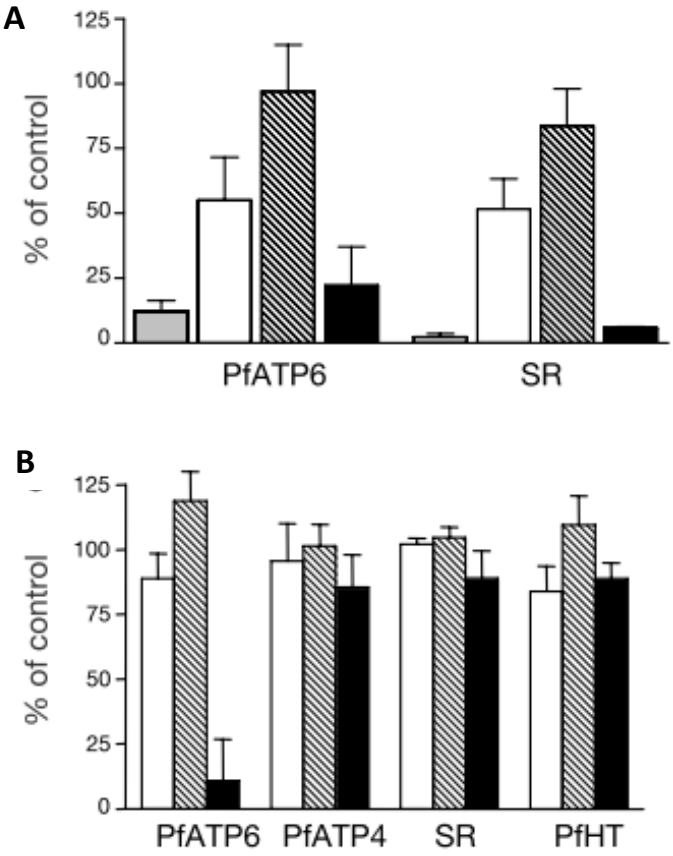

Figure 22 - Reported inhibition of the ATPase activity of SR and PfATP6, PfATP4 and PfHT expressed in X. laevis oocytes.

A) *Inhibition of SR and PfATP6 with classical SERCA inhibitors. White bar – sodium orthovanadate 100 µM, grey bar – thapsigargin 0.8 µM, black bar – CPA 1 µM, hatched bar – ouabain 100 µM.* ***B)*** *Inhibition of PfATP6, PfATP4, PfHT and SR with several antimalarials. White bar – quinine 10 µM, hatched bar – chloroquine 1µM, black bar – artemisinin 1 µM for PfATP6 and 50 µM for the other transporters* (Eckstein-Ludwig et al., 2003).

A mutual antagonism between artemisinin and thapsigargin on *in vitro* cultures of *P. falciparum,* when administrated simultaneously, was reported. Fluorescent thapsigargin (BODIPY-thapsigargin) was localized in inner structures of parasites (Figure 23 a) while competition with artemisinin was reported to reduce BODIPY-thapsigargin binding (Figure 23 c). The addition of deferrioxamine (a Fe^{2+} chelator) was reported to abolish the competition of artemisinin for BODIPY-thapsigargin binding sites (Figure 23 d), suggesting a role of iron on artemisinin activity. Fluorescent-artemisinin distributed alike BODIPY-thapsigargin in infected erythrocytes, to inner membranous structures, but

not in the food vacuole (Figure 23 g). With these various arguments, the authors claimed to have discovered the artemisinin target: PfATP6 (Eckstein Ludwig 2003). Concerning these images one can already point out that they do not demonstrate what the authors claim to prove. There is no evidence that artemisinin, nor thapsigargin, are localizing on the ER due to the lack of a specific labeling of this structure. This can be localized to the food vacuole, for instance. When compared to the localization in live parasites, this seems very different and there is a clear staining in the cytosol, and other unknown structures indicated with arrows (Figure 23 g).

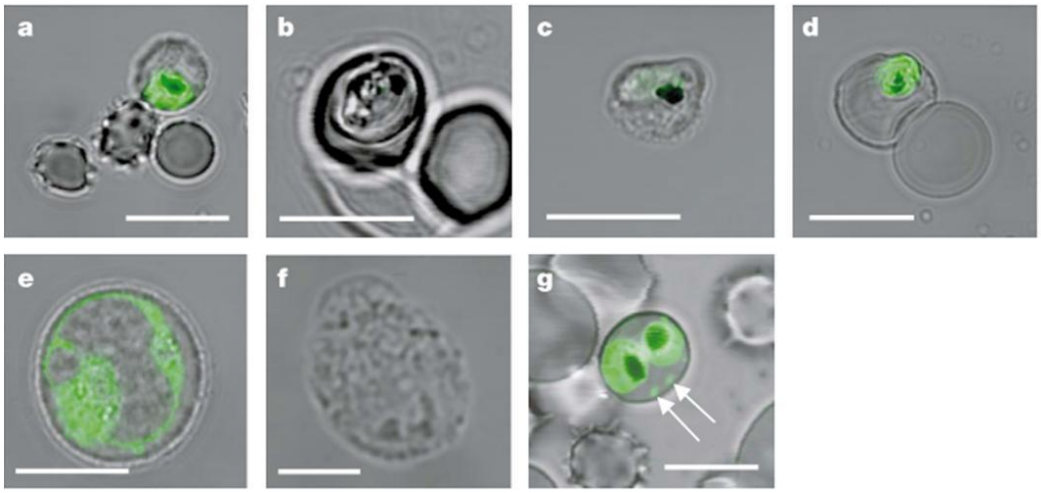

Figure 23 - Localization of BODIPY-thapsigargin and fluorescent artemisinin, in P. falciparum infected erythrocytes.

a) *BODIPY-thapsigargin labeling in infected erythrocytes,* ***b)*** *BODIPY-thapsigargin labeling in presence of 50 µM of thapsigargin,* ***c)*** *BODIPY-thapsigargin labeling in presence of 50µM of artemisinin,* ***d)*** *BODIPY-thapsigargin signal after pre-incubation in 50 µM artemisinin and 100 µM desferrioxamine;* ***e)*** *BODIPY-thapsigargin distribution in the lymphocyte cell line PM-1, showing typical SERCA-type distribution;* ***f)*** *BODIPY-thapsigargin signal in PM-1 cell after competition with 50 µM of unlabelled thapsigargin;* ***g)*** *Distribution of labeled artemisinin in live parasites (Arrows indicate artemisinin in erythrocyte cytosol). White scale bar representes approximately 30 µm* (Eckstein-Ludwig et al., 2003).

S. Krishna group expressed in a yeast whole cell system, PfATP6 wild-type and several mutants previously reported to be artemisinin insensitive (Pulcini et al., 2013). This study was achieved using a host yeast strain (K667) lacking one of the two Ca^{2+} ATPase genes *PMC1*, while the other gene *PMR1* could be inactivated by absence of calcineurin. Strain K667 non transformed can only grow without high calcium concentration in the medium. They reported that the growth of yeast K667 is restored when transformed with pfatp6 in medium supplemented with $CaCl_2$. Growth of K667 transformed with pfatp6 was reported to be inhibited by artemisinin and derivatives, CPA but not deoxyartemisinin (inactive artemisinin that lacks endoperoxide bridge) (see Figure 24). These results were described as “very significative” (represented with three stars on Figure 24). However, only CPA seems to present a real inhibition of yeast growth. Deferoxamine increased inhibition of artemisinin, suggesting a role of iron in the activity of this antimalarial. Introduction of mutations L263E, A623E,

S679N and A623E/S679N (reported to confer PfATP6 resistance to artemisinin) in the yeast strain K667 restored growth on 20 mM calcium, but failed on 30 mM calcium. Similar "significant" effect of artemisinin was observed with mutant S679N and A623E/S679N (Pulcini et al., 2013).

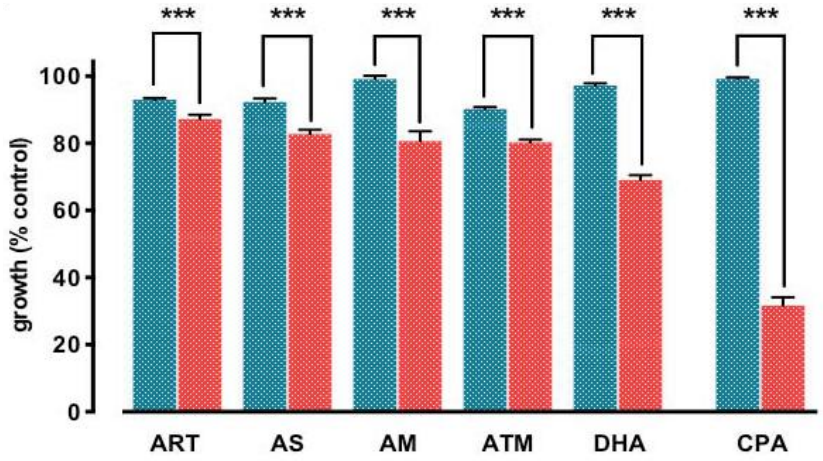

Figure 24 - Inhibition of in vitro growth of yeast K667::pUGpd (blue bars) and K667::pfatp6 (red bars) by different antimalarials.

*Yeast growth is expressed as percentage of control cultures (growth of each strain in absence of the drug). 10 µM of drug, data are mean ± SEM values of at least 9 replicates (3 independent biological experiments) (***P<0.001). pUGpd – empty vector. Yeast strains transformed with the empty vector (K667::pUGpd) were grown in low calcium, unlike yeast K667::pfatp6 (CaCl2 at 40 mM), because the first was unable to grow in high calcium due to the absence of a calcium ATPase* (Pulcini et al., 2013).

II.7.4 – Mutations in PfATP6, responsible for artemisinin resistance

Reports of an *in silico* model of PfATP6 were elaborated from SERCA1a crystallographic structure and an analysis of the binding site of thapsigargin (between helices M3 and M7, with loop L6 and L7 forming the apex of the pocket). Both proteins revealed several amino-acid differences in this region such as Leucine 263, and the equivalent position Glutamic acid 255 in SERCA1a. Krishna and collaborators mutated PfATP6 in L263E and expressed this mutant in *X. laevis* oocytes (Uhlemann et al., 2005). They found that this residue was responsible for artemisinin sensitivity and that this mutant was no longer inhibited by artemisinin. Similar results were found for *P. berghei* PbSERCA when this protein was mutated to L263S. Opposite results were observed when *P. vivax* PvSERCA was mutated L263A (leucine and alanine are both non-polar neutral amino-acids with hydrophobicity values from 3.8 to 1.8, respectively), this mutation increased considerably the sensitivity to artemisinin. Mammalian SERCA1a was described to be insensitive to artemisinin (Eckstein-Ludwig et al., 2003) and possesses, in the equivalent position, a glutamate Glu255 that, when mutated into a leucine, SERCA1a would become sensitive to these antimalarials. With this study they claimed to have discovered the unique residue in PfATP6 (L263) responsible to confer artemisinin resistance when mutated.

In 2012 Krishna et al. published a corrigendum where they added data points not included in the original publication and standardized the analysis. They claimed to confirm that mutations L263D, L263E and L263K in PfATP6 abolish the sensitivity to artemisinin (Figure 25 and Table 8) (Uhlemann et al., 2012), showing no clear inhibition by artemisinin. They also confirmed and showed the actual raw data for SERCA1a-E255L (Table 8), that was one of the mutations criticized earlier (Cardi et al., 2010b).

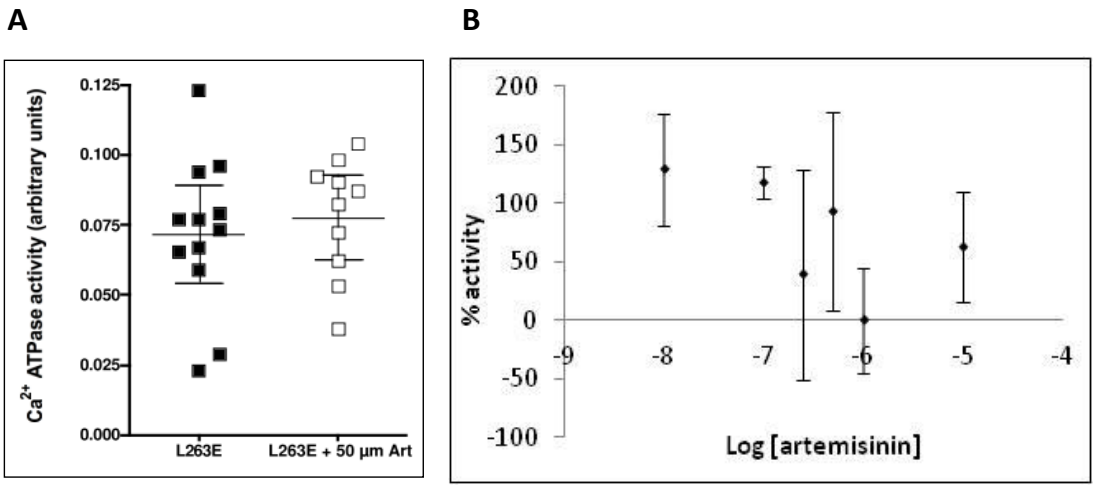

***Figure 25 - Effect of artemisinin on PfATP6 and SERCA1a expressed in X. laevis*.**

***A**- Effect of artemisinin on PfATP6-L263E mutant; **B** – Effect of artemisinin on SERCA1a-E255L. Data from* (Uhlemann et al., 2012).

	Eckstein-Ludwig et al., 2003;	Uhlemann et al., 2005	Uhlemann et al., 2012
Ki Artemisinin - PfATP6 wt (nM)	150	169 ±31	168.4 ± 54.4
Ki Thapsigargin - PfATP6 wt (nM)	146	n.d.	n.d.
Ki Artemisinin - PfATP6_L263E (nM)	n.d.	>50,000	>50,000
Ki Artemisinin - PfATP6_L263A (nM)	n.d.	63 ± 12	53.5 ± 12.3
Ki Artemisinin - PfATP6_L263S (nM)	n.d.	530 ±84	763.4 ± 327.1
Ki Artemisinin - PfATP6_L263Q (nM)	n.d.	552 ± 143	552.4 ± 201.7
Ki Artemisinin - PfATP6_L263D (nM)	n.d.	>50,000	>50,000
Ki Artemisinin - PfATP6_L263K (nM)	n.d.	>50,000	>50,000
Ki Artemisinin - PfATP6_F264L (nM)	n.d.	4 150 ± 1 850	7.2 ± 2
Ki Artemisinin - PfATP6_I89T (nM)	n.d.	122 ± 13	121.5 ± 22.4
Ki Artemisinin - SERCA1a_E255L (nM)	n.d.	314 ± 109	539
Ki Thapsigargin – SERCA1a wt (nM)	64	n.d.	n.d.
IC_{50} of Artemisinin - *P. falciparum* (nM)	5.47	4.4 ± 1.7	n.d.
IC_{50} of Thapsigargin - *P. falciparum* (µM)	n.d.	n.d.	2.55

Table 8-Comparison between the Ki values for artemisinin and thapsigargin inhibition of PfATP6 and SERCA1a and mutants expressed in X. laevis oocytes.

The values are given either in nM or in µM, as indicated, and issued from Krishna's group publications (Eckstein-Ludwig et al., 2003; Uhlemann et al., 2005, 2012). *n.d.– non determined.*

The authors tried to justify some lack of information by explaining that the magnitude of signal to noise in Ca^{2+}-ATPases activities does not always permit Ki determination, due to background activity, oocytes batches heterogeneity, and amount of heterologously expressed protein, contributing for large errors in individual experiments. Expressing more protein only increased background noise reducing the reliability of Ca^{2+}-ATPase activity measurements (Uhlemann et al., 2012). Moreover, it is known that controlling the level of expression of membrane proteins is a rather complicated task and needs modification of gene constructions and culture protocols, which were not described to have been modified. It is true that expressing proteins in *X. laevis* oocytes is not always an easy task and batches are rather heterogeneous, and that measuring ATPase activity directly from these membranes is a challenging task to overcome background ATPase noise. Then it is very tempting to conclude that these measurements were not sufficiently sensitive and accurate to affirm that PfATP6 is the sole directe target of artemisinins.

II.7.5 – PfATP6, is not the direct target of artemisinins

In 2010 a study of our laboratory clearly demonstrated that PfATP6 is not the direct target of artemisinins (Cardi et al., 2010b). Cardi and collaborators expressed PfATP6 in yeast and purified this protein by affinity chromatography, following the previously described procedure for SERCA1a (Jidenko et al., 2006). *Pfatp6* gene was codon optimized for yeast and most of the poly(A) or T tracts of the native sequence *pfatp6* gene were removed.

PfATP6 expressed in yeast and purified possessed a Ca^{2+}-dependent ATPase activity of 1.7 µmol of hydrolyzed ATP.min^{-1}.(mg of PfATP6)$^{-1}$ at pH 7.5, 25°C and in presence of 0.2 mg/ml $C_{12}E_8$ and 0.05 mg/ml DOPC. PfATP6 behaves like a mammalian SERCA, inhibited by vanadate and EGTA, and also by classical SERCA inhibitors (thapsigargin, CPA and BHQ) (Cardi et al., 2010b). At 25ºC PfATP6 was inhibited by BHQ (20 µM for 50% inhibition), CPA (3 µM for total inhibition) and thapsigargin (45 µM, instead of a nanomolar range for SERCA1a (Sagara and Inesi, 1991) (Figure 26). One may note that Krishna and collaborators initially reported that thapsigargin inhibited PfATP6 expressed in *X. laevis* oocytes at the nM range (Ki = 146 nM,(Eckstein-Ludwig et al., 2003)).

According to Uhlemann et al 2005, SERCA1a was reported to be insensitive to artemisinins but, as mentioned above, the mutant SERCA1a-E255L had the particularity to become sensitive to these antimalarials (Uhlemann et al., 2005). SERCA1a-E255L was expressed in COS cells and also expressed in yeast and purified as previously described (Jidenko et al., 2006). SERCA1a-E255L had a slightly smaller maximal ATP hydrolysis rate than the wild-type SERCA1a, but both proteins were equally inhibited by classical SERCA inhibitors (thapsigargin, CPA and BHQ) and EGTA (calcium chelator) but not by artemisinin (Cardi et al., 2010b).

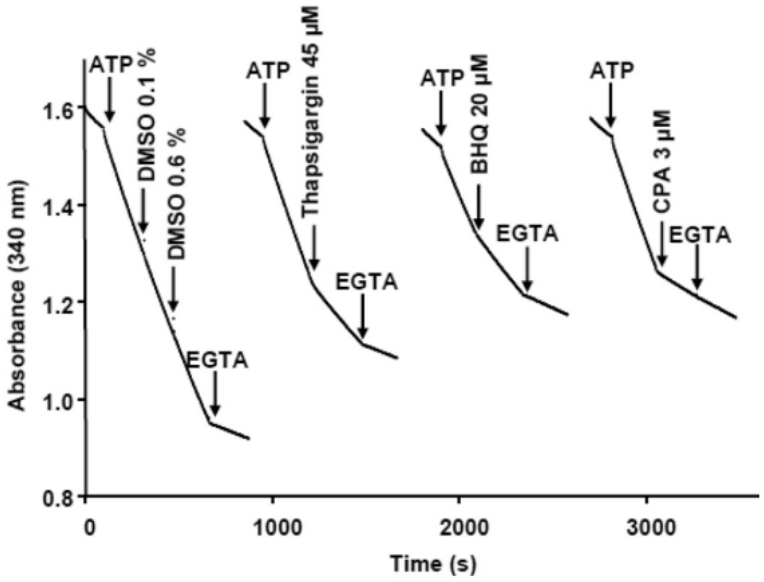

Figure 26 – PfATP6 Ca^{2+} dependent ATPase activity and inhibition by SERCA classical inhibitors.

These experiments were performed with 5 µg/ml of purified PfATP6 at 25°C and in presence of $C_{12}E_8$/DOPC (0.2/0.05 mg/ml). The reaction was triggered by the addition of 5mM ATP and stopped with 750 µM of EGTA. Source: (Cardi et al., 2010b).

In conclusion, both PfATP6 and SERCA1a-E255L, expressed in yeast and purified, were proven to be active calcium pumps. None of these proteins Ca^{2+}-dependent ATPase activity was inhibited by artemisinin or derivatives (up to 10 µM), at temperatures ranging from 20ºC to 37ºC, even with pre-incubation of PfATP6 with the antimalarials for 10 min and in the presence of 10 µM of Fe^{2+} (Figure 27). The difference between the purified PfATP6 and the PfATP6 expressed in oocytes membranes is the presence of detergent in the purified protein that can interact with the drugs. So PfATP6 was relipidated and the detergent completely removed using Bio-beads®, but even then artemisinin derivatives were unable to inhibit PfATP6, even in the presence of iron. Expression of PfATP6 in COS-1 cells was not successful (as previously noted (Eckstein-Ludwig et al., 2003). The expression in COS-1 cells also demonstrated that SERCA1a-E255L was not inhibited by artemisinin, and that E255L is not responsible for transforming SERCA1a into a protein sensitive to artemisinin. These data do not support a direct action of artemisinin on PfATP6. It does not exclude an indirect action or an upstream mechanism that could act upon PfATP6 (Cardi et al., 2010b), though it has still to be shown that artemisinin releases calcium from the ER store, as observed with CPA (Alleva and Kirk, 2001; Becker and Kirk, 2004).

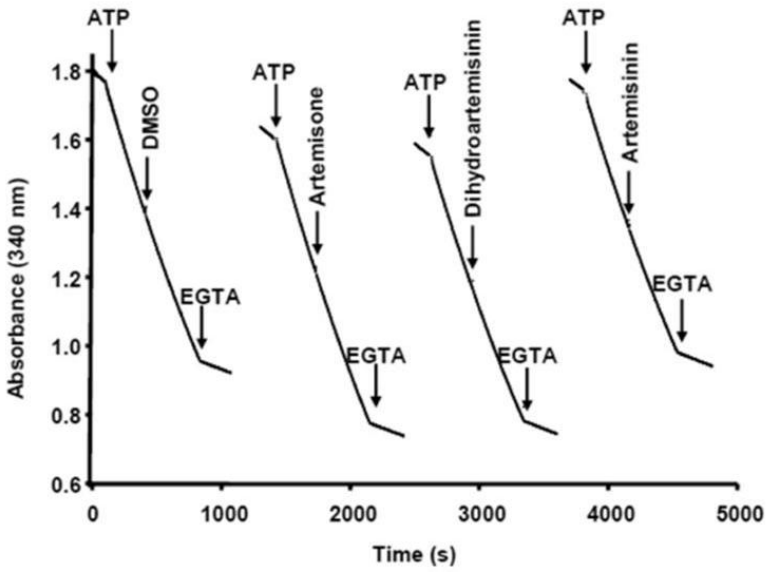

Figure 27 - PfATP6 Ca^{2+} dependent ATPase activity and inhibition by artemisinins.
These experiments were performed with 5 µg/ml of purified PfATP6 at 25°C and in presence of $C_{12}E_8$/DOPC (0.2/0.05 mg/ml). The reaction was triggered by the addition of 5mM ATP and stopped with 750 µM of EGTA. The tests were performed with 2µl of 10mM DMSO and 10 µM final concentration of each antimalarial. Source: (Cardi et al., 2010b).00000

In another posterior study of our laboratory, homology models of PfATP6 structure (Figure 20) showed no correlation with artemisinin binding, but the binding pocket of CPA was homologous between PfATP6 model and SERCA1a crystallographic structure (Arnou et al., 2011). CPA revealed itself to have higher affinity in inhibiting PfATP6 than SERCA1a (Cardi et al., 2010b). For this it has been suggested to be a good starting point for compound design or remodeling, if one is able to lower its toxicity for mammalian cells. It has been described that the key determinant amino acids of the binding pocket of CPA in PfATP6, when compared to SERCA1a is rather different. These differences can be exploited to design CPA derivatives specific inhibitors to PfATP6 (Kotšubei et al., 2013).

Although PfATP6 is not the direct target of artemisinins it remains a good potential target based on its homology to SERCA1a that has several inhibitors characterized to have a clinical-potential (Denmeade and Isaacs, 2005; Yatime et al., 2009), but it is still sufficiently different to be specifically targeted (eg., better affinity for CPA then SERCA1a), and because of the biological importance of SERCA proteins in calcium homeostasis (Arnou et al., 2011). Other evidences are the fruitless attempts of *PfATP6* knockout and complementation experiments in 3D7 lines, suggesting a genetic validation as an antimalarial target. These assays were unsuccessful; including complementing with wild-type or double mutated full length *pfatp6*, and other techniques. A double recombination construct failed to KO *pbSERCA* (*Plasmodium berghei* SERCA, malaria rodent model). All of these assays in trying to construct a *P. falciparum* KO for *pfatp6* were fruitless, suggesting that PfATP6 is essential for parasite survival (Pulcini et al., 2013).

II.7.6 – PfATP6 polymorphisms

As previously stated, after PfATP6 was proposed to be the direct target of artemisinins (Eckstein-Ludwig et al., 2003), the scientific community thought to have finally found the so long expected molecular marker to monitor the emergence of artemisinin resistance. However, no clear associations between mutations in *pfatp6* and artemisinin resistance were found. Several groups compared sequences of *pfatp6* between field isolates from distinct geographic regions (see section *I.4.4 – Molecular markers for artemisinin resistance*).

In 2010, *pfatp6* was sequenced from 100 field isolates before ACT deployment. A clear evidence for geographic structuring and *pfatp6* was found to be highly polymorphic (Jambou et al., 2010). A total of 33 SNPs were found with 19 non-synonymous substitutions (3 in the A domain, 21 in the N domain - 11 in the *Plasmodium*-specific sequence and 7 in P-domain and 5 in the residues essential for catalytic activity). No SNPs were found in the conserved SERCA domains (ATP, phosphate and Ca^{2+} binding).

Dahlström and coworkers sequenced *pfatp6* from 388 clinical isolates from different geographic origins, mainly from Zanzibar and Tanzania (Dahlström et al., 2008). They identified 33 SNPs, 29 new non-synonymous substitutions. Three new high frequency mutations in Zanzibar were described E431K, N569K and A630S, but no evidence of L263E neither S769N mutation. E431K, N569K, A630S were found at higher frequency. L263E mutation was never found in artemisinin resistant field isolates. But a mutation A623E on *pfatp6* was reported to have increased since ATC administration begun in Niger (Bacon et al., 2009). Resistance to artemether was significantly associated with *pfatp6* mutations A623E and S769N (Pillai et al., 2012). Other mutations on PfATP6 were reported, but several other studies showed no association between artemisinin resistance and these mutations (see Table 7).

In 2011, another study analyzed *P. falciparum* field isolates before implementation of ACTs, where 656 analyzed parasites were unexposed to artemisinins (Africa, Asia, Oceania and South America) and searched for polymorphisms in *pfatp6* (Tanabe et al., 2011). 64 single nucleotide polymorphisms (SNPs) were found, of which 43 were newly discovered (23 non-synonymous) and 38 were non-synonymous mutations. No isolates showed S769N or L263E substitutions, but the previously proposed artemisinin-associated mutation E431K was found in Africa, Asia and South America (Jambou et al., 2005; Legrand et al., 2008). Modification I89T and N465K, that were previously described to not be associated with resistance, were found in Asia and Oceania isolates (Dondorp et al., 2009). But it is important not to forget that *pfatp6* is characterized by abundant SNPs very different from one geographic region to another, and the majority is only observed at less than 5% frequency. The ratio between synonymous and non-synonymous mutation on *pfatp6* is close from neutrality (Wang et al., 2010). This makes particularly difficult the detection of *pfatp6* modifications linked to artemisinin resistance emergence (Tanabe et al., 2011; Woodrow and Bustamante, 2011). If the mutation arises independently in other geographic regions, the SNPs would be difficult to detect. However if this mutation is advantageous and linked to other SNPs, when selected, the same multi SNP background-region specific will be selected, and by selective sweep the genetic diversity of haplotypes will greatly decrease (Tanabe et al., 2011). Also, *pfatp6* seems to be localized in a poorly conserved region prone to polymorphisms that are non-adaptive, and hence positive selection is unlikely; and *pfatp4* (described as the target of spiroindolones, see below) is localized in a restricted conserved region (Gardner et al., 2011).

Finally, there is no clear evidence that *pfatp6* mutations have an effect on artemisinin resistance due to naturally high polymorphic genetic background (Imwong et al., 2010; Woodrow and Bustamante, 2011).

II.8 - PfATP4

In 1995 *P. falciparum* cation-translocase was described and called PfATPase 4. This protein was discovered in a study of the transmembrane pH gradient formation in the malaria parasite, which is involved in many processes such as chloroquine accumulation (Trottein and Cowman, 1995).

Pfatp4 is an AT-rich ~4 kb gene with no apparent introns, localized on chromosome 12. It is expressed immediately after parasite invasion of red blood cells, throughout the asexual cycle from early rings to trophozoites (detected by immunofluorescence and mRNA blots) but also in free merozoites (Krishna et al., 2001), but mRNA is no longer detected in later erythrocytic stages (Dyer et al., 1996). It contains a fragment of the pseudogene *pfatpase5.* PfATP4 was described as being located in discrete compartments at the periphery of the parasite, detected by immunofluorescence (Dyer et al., 1996). Later PfATP4-GFP was found to be localized to the parasite plasma membrane during erythrocyte cycle (Rottmann et al., 2010).

PfATP4 protein (1264 amino acids and 140 kDa) has a putative transmembrane organization similar to some other eukaryote and prokaryote P-ATPases with 10 predicted transmembrane domains, highly conserved phosphorylation sites and nucleotide-binding and transduction domains (Krishna et al., 2001).

Already in 1996, Dyer and coworkers described PfATP4 as being much more distant from mammalian SERCAs than PfATP6 (Dyer et al., 1996). But, by sequence homology it seemed that PfATP4 was close to Ca^{2+} - ATPases of the endoplasmic reticulum, in regions of functional importance, by conservation of a critical amino-acid involved in calcium binding in SERCA (Clarke et al., 1989a, 1989b; Dyer et al., 1996; Krishna et al., 2001; Trottein and Cowman, 1995; Trottein et al., 1995). The sequence amplified for the *pfatp5* pseudogene revealed strong similarities with Na^{+}/K^{+} ATPases, interrupted with multiple STOP codon. PfATP4 presented 43% identity to a Na^{+}/K^{+} ATPase and 30% homology with SERCA proteins (Jensen et al., 2006), however it did not present motifs for interaction with phospholamban nor calmodulin (features of SERCA and PMCA respectively) (Krishna et al., 2001).

II.8.1 – PfATP4 as a proposed Ca^{2+}-ATPase

Krishna and collaborators in 2001 obtained the full length sequence for *pfatp4* and expressed this protein in *X. laevis* oocytes (Krishna et al., 2001). They characterized PfATP4 as a Ca^{2+} - ATPase, distinct from SERCA pumps. It was reported to be stimulated by a broader range of calcium, resistant to ouabain inhibition and thapsigargin, but inhibited by vanadate and CPA. Its localization to the

parasite's plasma membrane suggested that it was probably closer to a PMCA than a SERCA pump (Dyer et al., 1996). According to these results, the authors excluded the hypothesis of being a Na^+/K^+ ATPase (PfATP4 was insensitive to ouabain), but it seemed also to not belong to any of the conventional Ca^{2+}-ATPases categories (Krishna et al., 2001). Krishna and co-workers suggested then a new P-type Ca^{2+} ATPase subfamily (according to Pittman's classification (Figure 15) (Pittman et al., 1999), which would group Apicomplexan orthologs of PfATP4, as no orthologs were found in non-Apicomplexan organisms (Krishna et al., 2001). This also led them to suggest that PfATP4 could be a good potential target, as apparently no orthologs were known at that time (Krishna et al., 2001).

II.8.2 – PfATP4 as the target of Spiroindolones

In 2010, a group of researchers found a promising class of antimalarials: the spiroindolones (Rottmann et al., 2010). They screened for a large library (12.000 natural and synthetic products, with structure resemblance found in natural products) on *P. falciparum in vitro* erythrocyte culture, by the whole cell approach (Chatterjee and Yeung, 2012). The first screening found 275 compounds with submicromolar antiparasitic activities from these only 17 hits were analyzed. The other were discarded because of toxicity on mammalian cells (IC_{50}>10µM), or because they were not active against multidrug resistant parasites. From these 17 hits, one compound stood out for pharmacological lead optimization, a synthetic compound related to spiroazepineindole class, with favourable pharmacological profile. The best optimized compound (NITD609) was further analyzed (Rottmann et al., 2010).

NITD609 revealed to be compatible with large scale manufacturing and conventional tablet formulation, safe (no cytotoxicity, genotoxicity, cardiotoxicity, mutagenic activity, nor binding to several G-coupled receptors, ion channels and enzymes tested), active against drug-resistant parasites, kill erythrocytic stages of *P. falciparum* (IC_{50} 0.5 – 1.4 nM) and of *P. vivax,* can be orally administered and has long half-life and good bioavailability (Rottmann et al., 2010).

At lower concentration schizonts were the most susceptible stage; thus the target was suspected to express during this stage. They selected for spiroindolones resistant strains, after submitting to NITD609 pressure a multi-drug resistant strain, described to be more prone for mutations (Dd2). The IC_{50} values raised but it stayed always in the nanomolar range, suggesting that spiroindolones did not trigger high-resistance *in vitro.* By high-density tiling array [16], they analyzed hybridization profiles and compared non-resistant to resistant strain. Using a strict cutoff, they have identified 27 genomic differences, 7 of these localized in *pfatp4* sequence. Sequencing of *pfatp4* revealed 11 non-synonymous mutations in resistant parasites, and low diversity among field isolates, although the residues required for cation translocation were not mutated. These data suggest that treatment with spiroindolones induces mutations in *pfatp4,* pointing for this P-ATPase being the target of these new antimalarial class. They also constructed transgenic *pfatp4* mutated D1247Y and I398F/P990R transgenic lines. These parasites possessed a higher IC_{50} (Rottmann et al., 2010).

[16] High-density tiling array - ~6 million single stranded 25-oligonucleotides complementary against *P. falciparum* genome, covering ~90% of coding regions and 60% of non-coding, with probes spaced every 2-3 nucleotides.

NITD609 is actually in phase IIA of clinical trials, and according to MMV it is the first molecule of the past 20 years that presents a different mechanism of action (http://www.mmv.org/research-development/rd-portfolio/).

Additionally, Rottmann and collaborators have been unable to reproduce the previous findings of Krishna et al. (2001), regarding PfATP4 Ca^{2+} dependent ATPase-activity (Figure 28). They also definitely ruled out that this protein was not localized on the endoplasmic reticulum by generating a PfATP4-GFP fusion that was co-transfected with mRFP-PfSec12 (an ER marker) into parasites, and PfATP4-GFP localized at the plasma membrane in *P. falciparum* intraerythrocytic stages (Rottmann et al., 2010).

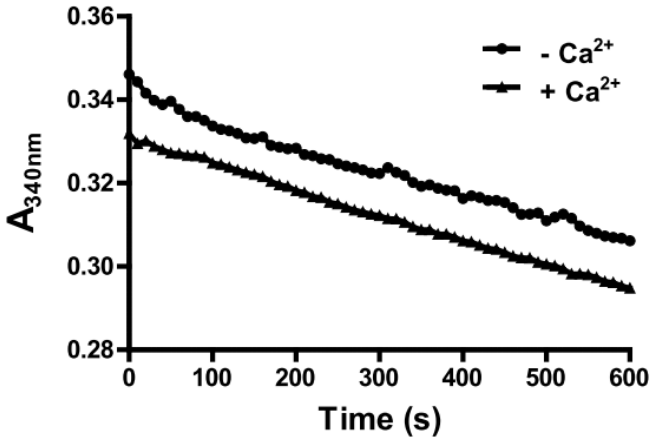

Figure 28– PfATP4 Ca^{2+} - dependent ATPase activity in X. laevis oocytes.
Source (Rottmann et al., 2010).

II.8.3 – PfATP4 is a Na^+/H^+ - ATPase

In 2013, members of the same group that discovered spiroindolones, demonstrated that, unlike it was suggested by Krishna and Dyer and collaborators reports (Dyer et al., 1996; Krishna et al., 2001), PfATP4 is in fact a Na^+ - ATPase (Spillman et al., 2013). However, it is importante to note that Dyer and coworkers proposed that PfATP4 was a Ca^{2+}-ATPase based on sequence homology results (Dyer et al., 1996), unlike Krishna and coworkers that affirmed that PfATP4 was a Ca^{2+}-ATPase based on Ca^{2+} - dependent ATPase activity measurements (Krishna et al., 2001).

They started by demonstrating a sequence homology between PfATP4 and ENA Na^+-ATPase [17] from lower eukaryotes such as other prokaryotes (*Saccharomyces, Leishmania, Trypanosoma, Entamoeba*). PfATP4 contains an eight amino acid motif conserved in ENA Na^+-ATPase - ^{849}IVQSLKRK

[17] ENA ATPase comes from *exitus natru*: exit of sodium. It is very similar to the SERCA pumps, and is probably a K^+ or Na^+ efflux ATPases. They are included in the P-type ATPase phylogenetic group IID (See Figure 15) (Spillman et al., 2013).

- especially the 854KRK, that is responsible for Na^+ transport, absent in SERCA and PMCA transporters (Spillman et al., 2013).

They functionally isolated trophozoites from the host infected erythrocyte by saponin treatment, permeabilizing the host membrane. They loaded this preparation with a dye that becomes fluorescent upon binding to Na^+. In presence of high concentrations of Na^+ (the parasite usually has a low intracellular $[Na^+]_i$), the inhibition by sodium orthovanadate, a P-ATPase inhibitor, increased $[Na^+]_i$; furosemide (protozoal Na^+ inhibitor) caused a little increase; but no effect was observed in the presence of ouabain (a Na^+/K^+ - ATPase inhibitor) neither with EIPA (a Na^+/H^+ exchanger inhibitor ethylisopropylamiloride). When spiroindolones (NITD246 and NITD139) were used on isolated trophozoites, a dose-dependent increase of fluorescent sensitive dye dependent of $[Na^+]_i$ occurred immediately (Spillman et al., 2013).

Spiroindolone NITD246 also induced cytosolic pH increase. This alkalination was dependent on the presence of high extracellular Na^+ concentration. This was the consequence of the inhibition of a Na^+/H^+ ATPase, leaving the cell with only a V-ATPase functioning, extruding constantly protons, increasing the intra-parasite pH (*Figure 29*). Application of concanamycin A (V-ATPase inhibitor) reverted this phenotype. The same way, NITD246 had no effect upon $[Ca]_i$, in contrast with the results described by Krishna et al 2001. But CPA caused an increase in $[Ca]_i$, consistent with the inhibition of PfATP6 present in the isolated trophozoites. Spiroindolones also disrupted a Na^+-dependent ATPase activity, affecting more importantly ATP hydrolysis in the infected erythrocytes, in presence of high $[Na^+]_i$ (*Figure 29*) (Spillman et al., 2013).

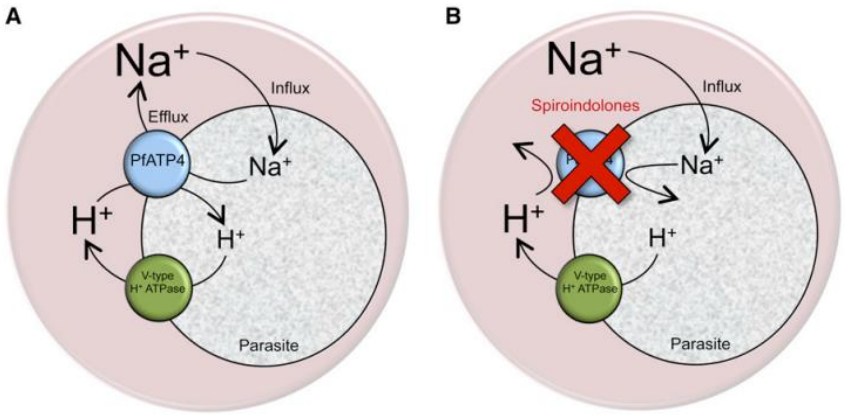

Figure 29– Proposed mechanism of spiroindolones on PfATP4

A- *Normal functioning of PfATP4 Na^+ / H^+- ATPase in high $[Na^+]$ medium. Intracellular Na^+ is extruded and H^+ imported and ATP hydrolysis, proton influx being balanced by V-type ATPase H^+ efflux.* ***B –*** *Inhibition of PfATP4 by spiroindolones leads to an increase in $[Na^+]_i$ by passive influx resulting from of the Na^+ gradient, in "high" $[Na^+]_e$ medium. The functioning V-type ATPases continue to extrude actively protons, provoking an alkalynation of the parasite cytosol. Source:* (Spillman et al., 2013).

PfATP4 mutant parasite lines selected for resistance to spiroindolones (Rottman et al 2010) had an altered Na^+ transport (Spillman et al., 2013). These lines showed a decreased sensitivity to

spiroindolones growth inhibition effect, and they were less affected for Na^+ transport disruption, in the presence of spiroindolones. The authors demonstrated that spiroindolones affected the $[Na^+]_i$ in infected erythrocytes, providing evidences of the disruption of Na^+ efflux. Spiroindolones provoke the rising of intracellular pH but $[Ca]_i$ remains unaltered (Spillman et al 2013).

II.9 – PfAdT

II.9.1 – The ATP/ADP carrier family

The physiological role of the ATP/ADP carrier (AAC) is to exchange ADP from the cytosol with ATP from the inner mitochondria, with an exchange stoichiometry of 1:1. It is solubilized as a monomer (Nury et al., 2008), but some groups consider it is functionally an oligomer. It is formed by 6 transmembrane α-helices, in a cone shape accessible from the cytoplasm (Pebay-Peyroula et al., 2003).

Each human exchanges the equivalent of his/her own mass of ATP every day (Pebay-Peyroula et al., 2003). The bovine and human isoforms 1 of the ADP/ATP carrier (AAC) share 90% of amino acid identity, whilst yeast and bovine share only 50%. Bovine AAC has been extensively studied due to its abundance in heart mitochondria, being an easy transporter to isolate for functional and structural studies (Pebay-Peyroula et al., 2003; Ravaud et al., 2012). The crystallographic structure of the bovine AAC (Pebay-Peyroula et al., 2003), and recently, the yeast AAC (Ruprecht et al., 2014) are known, but the human carrier is still unknown.

All AAC transporters possess a signature sequence essential for transport activity: RRRMMM, which is absent in other mitochondrial carriers (Pebay-Peyroula et al., 2003). These proteins are specifically inhibited by two classes of natural poisons: the atractyloside (ATR) and carboxyatractyloside (CATR), from *Atractylis gummifera* Mediterranean plant; and the bongkrekic acid (BA) and isobongkrekic acid (isoBA), from *Pseudomonas cocovenenans,* a pathogen that develops in coconut milk (Vignais and Lunardi, 1985; Vignais et al., 1976). Both N- and C- termini were demonstrated to be oriented towards the cytosolic regions (Brandolin et al., 1989; Pebay-Peyroula et al., 2003; Trézéguet et al., 2000).

ADP/ATP transport in AAC is pH sensitive and maximal when the pH lies between 6.5 and 7.5, (Ravaud et al., 2012; Broustovetsky et al., 1997). A single highly conserved amino acid residue, responsible for pH-dependent ADP/ATP exchange, was recently identified by Ravaud et al., (2012). The lysine 22 of the bovine AAC (K23 in hAAC) was demonstrated to be implicated in pH sensing and essential for transport. Mutation in K22 was shown to abolish AAC activity (Nelson et al., 1993; Pebay-Peyroula et al., 2003; Ravaud et al., 2012). The corresponding position in the *P. falciparum* ADP/ATP mitochondrial carrier (PfAdT) is K24 (Figure 30).

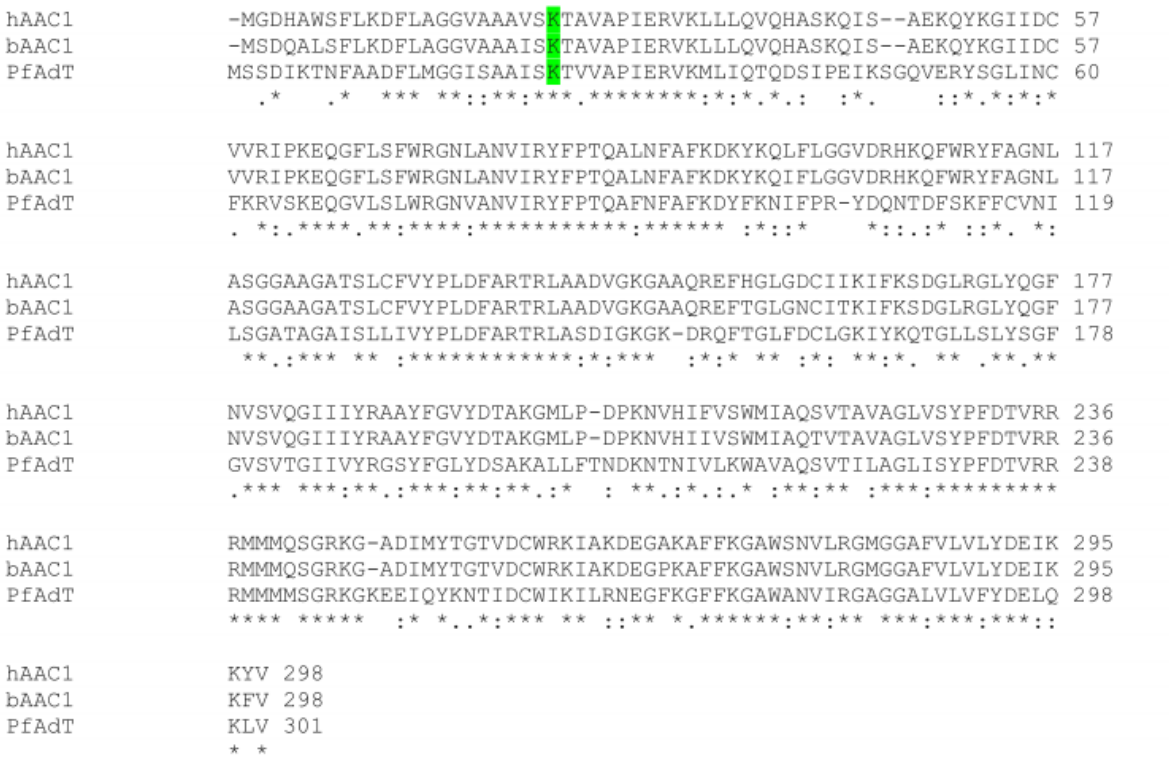

Figure 30 – *Alignment of human, bovine and P. falciparum mitochondrial AAC.*

hAAC1- human AAC isoform 1, bAAC1- bovine AAC isoform 1, PfAdT – P. falciparum mitochondrial AAC, or adenylate translocase. In green are the corresponding highly conserved lysines residues in the three organisms, described to be essential for AAC transport activity (Nelson et al., 1993; Pebay-Peyroula et al., 2003; Ravaud et al., 2012).

II.9.2 – The ATP/ADP carrier in *Plasmodium*

When *Plasmodium falciparum* infectes an erythrocyte, an increase in "new permeability pathways" occurs (Becker and Kirk, 2004; Kirk et al., 1994). A higher metabolism and solute transport necessary for parasite replication, triggers an increase in glucose and ATP consumption (Kanaani and Ginsburg, 1989). It was suggested that the parasite greatly contributes to supply the host erythrocyte in ATP by the mean of an ATP transporter localized on the parasite plasma membrane (Choi and Mikkelsen, 1990; Kanaani and Ginsburg, 1989). To achieve this supply from the ATP synthesis in the mitochondria to the host erythrocyte, an ADP/ATP carrier (AAC) is also present in the inner mitochondrial membrane and is called PfAdT (Hatin and Jaureguiberry, 1995).

The mitochondrial adenylate translocase of *P. falciparum* (PfAdT) is a transmembrane protein of 301 residues and 33.7 kDa, localized in the inner membrane of the mitochondria (Hatin and Jaureguiberry, 1995). PfAdT is constituted of 6 transmembrane domains, and N – and C - terminal regions are localized in the cytosolic region (Brandolin et al., 1989; Hatanaka et al., 1999; Majima et al., 1994; Trézéguet et al., 2000). It has a strong homology of 50.3 % with yeast and 61.2 % with

humans (Hatin and Jaureguiberry, 1995). It is coded by an A-T rich gene sequence of 903 bp, localized on *P. falciparum* chromosome 10 (Klingenberg et al 1985). PfAdT shares many features with animal AAC, and rather few with plants or yeast AAC, such as the ^{246}RKGK signature. But it presents the well conserved residues between species responsible for ATP and ADP binding (Arg237, Arg238, Arg 239 – corresponding to Arg252, Arg 253 and Arg 254 in yeast), and four conserved residues in membranes segments (Lys24, Arg83, Arg189, and Arg283 – respectively in yeast Lys38, Arg96, Arg204 and Arg294), are sites of interaction during nucleotide transport (Hatin and Jaureguiberry, 1995). Arg237, Arg238, Arg 239, in transmembrane fragments 5 and 6, were proven essential for translocation, and, as already mentioned above, Lys24 was proven essential for the transport activity in bAAC1 (Nelson et al., 1993; Pebay-Peyroula et al., 2003; Ravaud et al., 2012).

This transporter exchanges one molecule of cytosolic ADP with one molecule of ATP from the inner mitochondria lumen (Klingenberg, 1985). PfAdT is present in merozoites but strongly expressed in ring and schizont stages, increasing during trophozoites stage (mRNA detection) (Jambou et al., 1995).

II.9.3 – PfAdT as a potential antimalarial target

The mitochondrial anion carrier family includes the uncoupling protein (UCP), the phosphate carrier (PIC), the oxoglutarate carrier (OGCP), and the ADP/ATP carrier (AAC). These transporters are essential for good homeostasis. For instance, UCP1 is a target exploited against obesity amnd UCP2 is implicated in chronic inflammatory diseases (Rousset et al., 2004). The mitochondrial AAC is essential in eukaryotic cells. The lack of ADP/ATP transporter isoform 2 in yeast stops all aerobic growth. Moreover, dysfunctional ATP/ADP carrier is responsible for sever human diseases such as: myopathy, cardiomyopathy, encephalopathy or myoclonic epilepsy (Graham et al., 1997; Huizing et al., 1996). Mutation A123D in the human *SLC25A4* gene that encodes for the heart-muscle AAC1 is responsible for exercise intolerance, lactic acidosis, hypertrophic cardiomyopathy and mild myopathy in individuals carrying this mutation (Palmieri, 2008). A complete loss of ADP/ATP transport is observed when the hAAC1 mutant A123D is reconstituted in proteo-liposomes (Beyer and Nuscher, 1996). Senger's syndrome is an inherited autosomal recessive disease that expressed in congenital cataracts, hypertrophic cardiomyopathy mitochondrial myopathy and lactic acidosis. Although no mutations were found in gene *SLC25A4* coding for hAAC1, a reduction in hAAC1 expression was observed in these patients (Palmieri, 2008). In *P. falciparum,* when parasites are treated with bongkrekic acid, their maturation is inhibited (Trager, 1973).

It is difficult to isolate parasite-mitochondria that are not contaminated with human-mitochondria in sufficient amount to study. Also, the expression level of PfAdT is quite low in *Plasmodium* blood stages (Jambou et al., 1995). Purification from parasites is rather hard to achieve. To circumvent this issue, PfAdT was expressed in C43 (DE3) *Escherichia coli* strain enabling also the establishment of a system for inhibitors screening (Razakantoanina et al., 2008). *E. coli* does not possess any mitochondrial ADP/ATP transporter, which makes this cell a perfect system to study ATP transport across its outer membrane (Haferkamp et al., 2002). Furthermore, this transporter is directly accessible to potential inhibitors tested, unlike in *Plasmodium* where the compounds would need to cross several membranes before reaching the mitochondria (Narayanareddy, 1991). Bongkrekic acid

(a specific AAC transporter) inhibited this transport of radiolabelled ATP (IC_{50} < 1 µM), but atractyloside not (even at 100 µM) (Razakantoanina et al., 2008).

In this study the authors tested three crude extracts of plants from Madagascar, which have shown significant antiplasmodial activity: *Brachylaena ramiflora, Phyllarthron bernierianum, Strychnos sp.* Except for *Strychnos sp.* no inhibition of radiolabeled ATP uptake was observed, even though this plant extract has a comparatively lower affinity for PfAdT. A system for screening for PfAdT specific inhibitors was thus established (Razakantoanina et al., 2008).

To be able to produce PfAdT inhibitors that would be non-toxic for humans, one will have to explore the less than 40% of difference between these two orthologs. Although PfAdT has been previously described as being one of the most promising transporters to target for new antimalarial discovery (Staines et al., 2010): it would be interesting to meet this challenge.

III – The project

The goal of this PhD thesis was to study *Plasmodium falciparum* membrane transporters as potential antimalarial targets. In a first approach we aimed to give continuity to the work initiated by Delphine Cardi during her PhD thesis regarding PfATP6, the SERCA of *P. falciparum.* In parallel we attempted to initiate the study of a novel P-type ATPase: PfATP4, a recently proven Na^+/H^+ ATPase of *P. falciparum.* At last we intended to start the study of the ADP/ATP mitochondrial transporter of *P. falciparum.*

PfATP6 was previously expressed in yeast and purified by Delphine Cardi (Cardi et al., 2010b). This earlier project brought insights into the functional and biochemical characterization of this endoplasmic reticulum Ca^{2+}-ATPase. During this PhD project we aimed to go further:

1. In a first step we aimed to optimize the previous protocol for large scale purification of PfATP6, compatible with procedures for compounds screening.

2. We planned to establish a solid collaboration between three specialized partners to undertake new PfATP6 inhibitors discovery and evaluate their antiplasmodial activity.

3. To determine blood-stage specificity of the best compounds issued from the previous screening, we had first to determine the *P. falciparum* stages in which PfATP6 expresses. For this we have chosen to perform immunofluorescence technique with a PfATP6 specific antibody.

4. In parallel we attempted to express PfATP6, SERCA1a and SERCA1a-E255L mutant in an alternative system: *Xenopus laevis* oocytes membranes, as previously described by Krishna and collaborators (Krishna et al 2001, Eckstein-Ludwig et al 2003). These experiments were performed in collaboration with Dr. Hanne Poulsen, Dr. Michael Jakob Clausen, Dr. Jesper Møller and Dr. Poul Nissen. We planned to reproduce the experiments described in Eckstein-Ludwig et al 2003, and Uhlemann et al 2005, and verify if PfATP6 and the SERCA1a-E255L mutant (described as artemisinin sensitive) were inhibited by artemisinin, a very controversial point of view that these authors never stopped defending through years.

PfATP4 arouse our interest because it was described as a plasma membrane Ca^{2+}-ATPase (Krishna et al 2001, Dyer et al 1996), but also target of spiroindolones, one of the most promising future antimalarials (Rottman et al 2010). As achieved with PfATP6, we first attempted to introduce *pfatp4* gene into the yeast expression vector that would enable further purification of this transporter. Unfortunately, after more than a year of fruitless constant attempts, we decided to concentrate our efforts in other projects. In 2013, PfATP4 was finally described as being a Na^+/H^+ ATPase (Spillman et al 2013).

At this point we decided to introduce a new transporter to this project. In collaboration with Pr. Isabelle Florent, who previously participated in the establishment of the protocol for PfAdT inhibitors screening (Razakantoanina et al 2008), and combining the expertise in our laboratory and the laboratory of Pr. E. Pebay-Peyroula and Dr. S. Ravaud, for expression and purification of the human ADP/ATP carrier (hAAC), we decided to study PfAdT as a potential antimalarial target. We intended to study PfAdT wild-type and a predicted inactive mutant K24I (Ravaud et al., 2012)

1. We have introduced *pfadt_wt* and *pfadt_k24i* into the yeast expression vector and proceeded to expression of this protein in yeast, for a future crystallization project.

2. We have also introduced *pfadt_wt* and *pfadt_k24i* into a pET20b vector and expressed these proteins in *E. coli* strain C43 (DE3) (Miroux and Walker, 1996), for functional studies and inhibitors research.

In the following sections of this PhD thesis, we describe the results we obtained regarding the aims we established, along with the articles that were published or submitted in scientific journals. The discussion of these results inserted in their scientific context, published works and previous findings, is integrated into the Results and Discussion section, and follows our obtained data. Finally, a Conclusion and Perspectives of the work that was developed during these three years closes this thesis.

Results and Discussion

Chapter I

Large Scale production of PfATP6 in view of screening of potential antimalarial compounds

I.1 – Preamble

As presented above, large-scale production of PfATP6 enabled us to screen for new specific inhibitors in collaboration with Pcovery Company (Copenhagen, Denmark). Prior to the beginning of this project, the establishment of a procedure for expressing a recombinant form of PfATP6 in yeast and subsequent affinity purification, was the subject of Delphine Cardi's PhD in 2010 (Cardi et al., 2010a, 2010b); and recombinant SERCA1a too, leading to its crystallographic structure (Jidenko et al., 2005). For the first time it was possible to purify an active form of PfATP6 that could be biochemically characterized and inhibited by specific SERCA inhibitors (Arnou et al., 2011; Cardi et al., 2010b). PfATP6, due to its biological importance as a SERCA transporter and the fruitless attempts to knock-out this gene from *Plasmodium falciparum* (Pulcini et al., 2013), has been considered to be a good potential drug target (Arnou et al., 2011).

We aimed to establish a collaboration between three partners with different expertise areas. A first partner (us) able to heterologously express in yeast and purify an active form of PfATP6; a second partner that possessed a P-type ATPases inhibitors library and able to test a large amount of compounds on purified PfATP6 (Pcovery, Denmark); and a third partner that could culture *in vitro* blood-stages of *Plasmodium falciparum,* in view of testing the best compounds for their antimalarial activity, as well as verifying their toxicity on mammalian cells (Isabelle Florent, MNHN, France).

We intended to modify and establish an optimized protocol for over expression and purification of PfATP6 to prepare larger amounts of protein for screening purposes. Introducing a device to facilitate yeast breaking, the planetary mill pulverisette (Fritsh), enabling a more efficient, more homogenous, time and effort reducing procedure. This step is crucial for a good yield of membrane preparation, significantly decreasing the amounts of unbroken yeast. During the solubilization and batch purification of PfATP6 by streptavidin-Sepharose chromatography, several steps were modified from the previous protocol (such as: the washing of the light membrane fraction, the composition of some buffers, the mode of binding of the solubilized solution to the resin, and the mode of elution and concentration of the purified proteins).

We verified the activity of the newly purified PfATP6 by testing classical SERCA inhibitors (thapsigargin (thg), 2,5-di(tert-butyl)-1,4-benzo-hydroquinone (BHQ) and cyclopiazonic acid (CPA)). We verified that CPA was the most efficient inhibitor of PfATP6, as previously described (Arnou et al., 2011).

With the establishment of this protocol, Pcovery Company could screen for new PfATP6 inhibitors from its chemical library and provide with the best hits.

The best candidate molecules were then tested on *in vitro* erythrocyte cultures of *P. falciparum* and for their cytotoxicity, in collaboration with Isabelle Florent (MNHN, Paris, France). For this, as we had previously tested SERCA classical inhibitors on purified PfATP6 (Arnou et al., 2011; Cardi et al., 2010b), we decided to test these well-known molecules and assess if either a correlation exists between the enzymatic inhibition of PfATP6 and the antiplasmodial activity results. We established an experimental procedure with these compounds.

Some of these results were published in FEBS Journal in 2013 (David-Bosne et al., 2013). The article is presented here integrally, followed by the description of posterior experiments achieved in view of testing the cytotoxicity of the compounds. After this publication we were offered the opportunity of a new collaboration, an Italian group had 4-aminoquinoline/clotrimazole based antimalarials to test on purified PfATP6. For this we established an ATPase activity measurement protocol, allowing the measurement of several compounds consuming less enzyme.

In parallel we also aimed to improve the production of PfATP6 by: i) optimizing the yeast expression protocol; ii) and by changing the elution cleavage site from thrombin to TEV (Tobacco Etch Virus) protease.

I.2 - Article - Antimalarial screening via large-scale purification of Plasmodium falciparum Ca^{2+}-ATPase 6 and in vitro studies

Stéphanie David-Bosne [1], Isabelle Florent [2], Anne-Marie Lund-Winther [3, 4, 5], John B. Hansen[3], Morten Buch-Pedersen [3], Paul Machillot [1], Marc le Maire [1] and Christine Jaxel [1]

[1] UMR 8221 CNRS, Université Paris-Sud and CEA, Gif-sur-Yvette, France
[2] UMR 7245 CNRS, Team Adaptation of Protozoa to their Environment, National Museum of Natural History, Paris, France
[3] Pcovery Aps, Frederiksberg, Denmark
[4] Center for Membrane Pumps in Cells and Disease – PUMPKIN, Aarhus University, Denmark
[5] Department of Molecular Biology and Genetics, Aarhus University, Denmark

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Antimalarial screening via large-scale purification of *Plasmodium falciparum* Ca^{2+}-ATPase 6 and *in vitro* studies

Stéphanie David-Bosne[1], Isabelle Florent[2], Anne-Marie Lund-Winther[3,4,5], John B. Hansen[3], Morten Buch-Pedersen[3], Paul Machillot[1], Marc le Maire[1] and Christine Jaxel[1]

1 UMR 8221 CNRS, Université Paris-Sud and CEA, Gif-sur-Yvette, France
2 UMR 7245 CNRS, Team Adaptation of Protozoa to their Environment, National Museum of Natural History, Paris, France
3 Pcovery Aps, Frederiksberg, Denmark
4 Centre for Membrane Pumps in Cells and Disease – PUMPKIN, Aarhus University, Denmark
5 Department of Molecular Biology and Genetics, Aarhus University, Denmark

Keywords
antiplasmodial activity; Ca^{2+}-ATPase activity; PfATP6; recombinant protein; *Saccharomyces cerevisiae*

Correspondence
C. Jaxel, UMR 8221 CNRS, Université Paris-Sud and iBiTec-S, CEA Saclay, Gif-sur-Yvette F-91191, France
Fax: +33 1 6908 8139
Tel: +33 1 6908 3379
E-mail: christine.jaxel@cea.fr

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The most severe form of human malaria is caused by the parasite *Plasmodium falciparum*. Despite the current need, there is no effective vaccine and parasites are becoming resistant to most of the antimalarials available. Therefore, there is an urgent need to discover new drugs from targets that have not yet suffered from drug pressure with the aim of overcoming the problem of new emerging resistance. Membrane transporters, such as *P. falciparum* Ca^{2+}-ATPase 6 (PfATP6), the *P. falciparum* sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), have been proposed as potentially good antimalarial targets. The present investigation focuses on: (a) the large-scale purification of PfATP6 for maintenance of its enzymatic activity; (b) screening for PfATP6 inhibitors from a compound library; and (c) the selection of the best inhibitors for further tests on *P. falciparum* growth *in vitro*. We managed to heterologously express in yeast and purify an active form of PfATP6 as previously described, although in larger amounts. In addition to some classical SERCA inhibitors, a chemical library of 1680 molecules was screened. From these, we selected a pool of the 20 most potent inhibitors of PfATP6, presenting half maximal inhibitory concentration values in the range 1–9 μM. From these, eight were chosen for evaluation of their effect on *P. falciparum* growth *in vitro*, and the best compound presented a half maximal inhibitory concentration of ~ 2 μM. We verified the absence of an inhibitory effect of most of the compounds on mammalian SERCA1a, representing a potential advantage in terms of human toxicity. The present study describes a multidisciplinary approach allowing the selection of promising PfATP6-specific inhibitors with good antimalarial activity.

Introduction

Malaria is a life-threatening disease caused by an infection by parasites of the genus *Plasmodium* transmitted by female mosquitoes of the genus *Anopheles*. *Plasmodium falciparum* is responsible for the most

Abbreviations
BAD, biotin acceptor domain; BHQ, 2,5-di(tert-butyl)-1,4-benzo-hydroquinone; $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether; CPA, cyclopiazonic acid; DDM, *N*-dodecyl-D-maltoside; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; IC_{50}, half maximal inhibitory concentration; PfATP6, *Plasmodium falciparum* Ca^{2+}-ATPase 6; PMSF, phenylmethanesulfonyl fluoride; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; TES, 2-[2-hydroxy-1,1-bis(hydroxy-methyl)ethyl]aminoethanesulfonic acid.

severe form of human malaria. The absence of a vaccine makes drug treatment the only way to control malaria. According to the *World Malaria Report* of 2011, the estimations for 2010 were 216 million cases of malaria and 655000 deaths [1]. The World Health Organization (WHO) recommends the use of artemisinin-based combination therapies as the first-line treatment for uncomplicated malaria [2]. The increase of multiple resistances to classical antimalarials (e.g. quinolines and antifolates derivates) is becoming a major issue for public health [3,4]. Moreover, a decrease of sensitivity to artemisinin has already been reported in Western Cambodia [5,6]. To anticipate the emergence of new resistances, there is an urgent need to discover new targets and molecules with antimalaria effects.

The malaria parasite is a complex cell that contains (besides the plasma membrane) several membranes delimiting organelles such as the nucleus, endoplasmic reticulum, Golgi apparatus, vestigial plastid-like apicoplast, mitochondria and food/digestive vacuole [7]. These membranes contain several transporters that are responsible for essential cellular processes such as the movement of solutes across biological membranes, the regulation of essential nutrient uptake, ion homeostasis and the disposal of toxic waste, although transporters may also be part of the regulatory pathways [3,7]. Membrane transporters such as P-type ATPases are already utilized as specific and potent drug targets in several human diseases [7–9], irrespective of their location on the plasma membrane or in intracellular membranes [10]. Transporters therefore hold great potential as drug targets and have been largely exploited; for example, *P. falciparum* chloroquine resistance transporter [11,12], P-glycoprotein homologue 1 [7,11,13,14], *P. falciparum* multidrug resistance-associated protein [15,16] and ATP/ADP translocase [7,17].

Plasmodium falciparum Ca^{2+}-ATPase 6 (PfATP6) is the *P. falciparum* sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump, a member of the P-type ATPase family [18]. It is a membrane transporter considered to be localized on the parasite's endoplasmic reticulum and to play a crucial role in calcium homeostasis, and thus is expected to be essential for parasite growth and survival. The PfATP6 pump could therefore constitute a promising drug target for the treatment of malaria [18]. PfATP6 is a protein of 139 kDa with 52% sequence identity to human SERCA1a and 51% sequence identity to rabbit SERCA1a, although it has different functional properties compared to its mammalian orthologue: PfATP6 presents a lower affinity for thapsigargin and 2,5-di(tert-butyl)-1,4-benzo-hydroquinone (BHQ) and a much stronger affinity for cyclopiazonic acid (CPA), which comprise three classical SERCA inhibitors [18]. PfATP6 has previously been proposed as a target of the antimalarial class of artemisinin on the basis of the inhibition of PfATP6 Ca^{2+}-ATPase activity in *Xenopus* oocytes membranes heterologously expressing PfATP6 [19]. However, Cardi *et al.* [20] demonstrated that PfATP6, heterologously expressed and purified from yeast membranes, was insensitive to artemisinin [20].

Nevertheless, a number of early studies reported that specific mutations of the *PfATP6* coding sequence (L263E and S769N) might confer resistance to artemisinin and its derivatives [21,22], although subsequent work has provided no evidence of any causal relationship [23]. Chavchich *et al.* [24] and Cui *et al.* [25] have also demonstrated that there is no evidence supporting the involvement of PfATP6 in artemisinin responsiveness. Tanabe *et al.* [26] demonstrated a natural and random occurrence of numerous single nucleotide polymorphisms on *PfATP6*, showing that this gene is naturally very polymorphic [26]. Furthermore, the PfATP6 mutations were located in poorly conserved regions of the protein, consistent with simple genetic drift [27]. These findings indicated that the mutations found on *PfATP6* of *P. falciparum* strains presenting a diminished sensitivity to artemisinin may not correspond to a selective pressure of this drug on PfATP6.

Finally, recent genome-wide association studies have examined large numbers of field isolates in areas of emerging artemisinin resistance; these provide very powerful data that are highly relevant for testing whether PfATP6 could be involved in artemisinin action and resistance. Cheeseman *et al.* [28] specifically examined the PfATP6 locus in the context of emerging resistance in Western Thailand, whereas Takala-Harrison *et al.* [29] examined resistance in Western Cambodia. Both studies found no evidence for selection at the PfATP6 locus.

In summary, we consider that PfATP6 is susceptible to becoming a drug target as a result of its crucial role in cell biology, and not because it is the enzyme targeted by artemisinin. To identify new molecules that inhibit PfATP6, in the present study, we report a collaborative work that combines expertise in the heterologous expression in yeast and purification of PfATP6; ATPase activity measurements on purified PfATP6; drug screening from libraries of molecules; and, finally, testing of some of the best candidate molecules on the erythrocytic stage of *P. falciparum* cultures.

Results and Discussion

In vitro enzymatic inhibition and antiplasmodial activity of classical SERCA inhibitors

In previous studies, we expressed PfATP6 using a heterologous expression system in yeast. The protein was coupled to a biotin acceptor domain (BAD), which allows an *in vivo* biotinylation in yeast. The membrane fractionation gave rise to a light membrane fraction called P3, which contains PfATP6. The fraction was solubilized using the detergent *N*-dodecyl-D-maltoside (DDM). The protein was purified using a streptavidin resin, which is highly specific for biotinylated proteins. PfATP6 purified by this way is functional in terms of ATPase activity [20]. This enzymatic activity was measured spectrophotometrically using a coupled enzyme assay [30,31]. The effects of various mammalian SERCA1a inhibitors were investigated on PfATP6 to compare their effects on both proteins. We found that PfATP6 is less sensitive to thapsigargin and BHQ than rabbit SERCA1a, whereas CPA is a very powerful inhibitor of PfATP6 activity. This suggests significant and important differences between *P. falciparum* and mammalian proteins. It was possible to monitor the structural changes induced by drug binding on PfATP6 using tryptophan fluorescence measurement techniques [18,20]. These results are summarized as the half maximal inhibitory concentration (IC_{50}) PfATP6 in Table 1.

The first objective of the present study was to test the effect of these classical SERCA1a inhibitors on *P. falciparum* growth *in vitro*. We wanted to validate this procedure and test whether a correlation exists between the enzymatic inhibition of PfATP6 and the antiplasmodial activity results. Accordingly, we checked these compounds for their ability to inhibit the growth of two different *P. falciparum* strains (differentially sensitive to chloroquine: 3D7 and FcB1), which were maintained in culture as previously described by Trager and Jensen [32]. Drugs were assayed by using the semi-automated microdilution technique of Desjardins *et al.* [33], which consists of growing the parasites in 96-well plates, in the presence of serially diluted concentrations of tested drugs, for 48 h. One such experiment is shown for CPA in Fig. 1A. All three molecules inhibited parasite growth in erythrocytes, with IC_{50} values in the micromolar range, and with an approximately two-fold difference between the two strains, where 3D7 is less sensitive than FcB1.

As shown in Table 1, CPA has a more pronounced antiplasmodial activity than BHQ on both strains. This is also true when we compare the inhibitory effect of CPA on purified PfATP6 with that of BHQ [18,20]. The results demonstrate some correlation between the antiplasmodial activity of these two compounds and their enzymatic inhibition of PfATP6, whereas this is not the case for thapsigargin, which appears to be as toxic to parasites as CPA, with this being the case for

Table 1. Effect of SERCA classical inhibitors upon *P. falciparum* growth *in vitro*. IC_{50} PfATP6: values of the inhibition of the ATPase activity determined on the purified PfATP6 [18]. Indication of the IC_{50} values for each drug on two *P. falciparum* laboratory strains differently resistant to chloroquine (3D7 and FcB1). Values (μM) correspond to at least three biological replicates. ND, not determined.

Compounds	IC_{50} PfATP6	IC_{50} FcB1	IC_{50} 3D7
Thapsigargin	150	6.1 ± 1.05	11.5 ± 5.7
BHQ	65	17.2 ± 11.3	30.6 ± 13.5
CPA	0.4	4.9 ± 0.9	8.8 ± 3.4
Chloroquine	ND	0.13 ± 0.02	0.012 ± 0.002

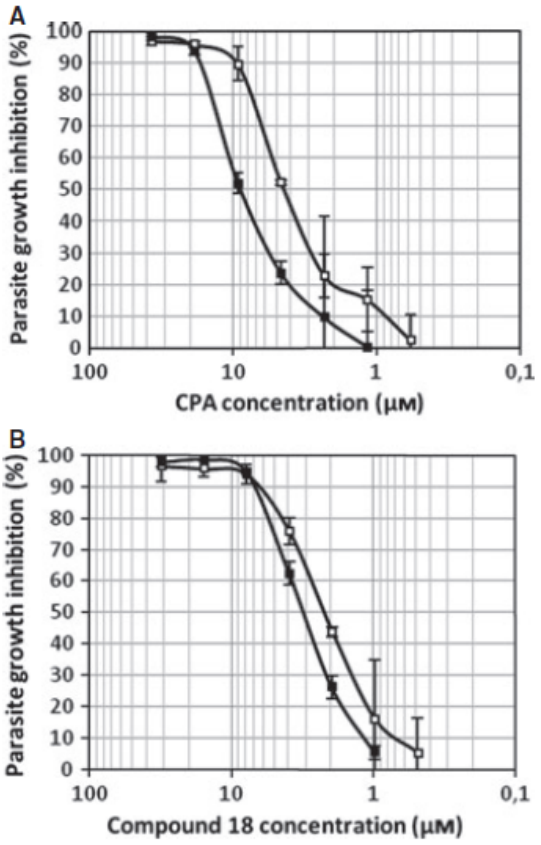

Fig. 1. Dose–response curves for CPA and compound 18 acting on *P. falciparum* viability. Effect of (A) CPA and (B) compound 18 on parasite growth inhibition. Compounds were tested on both FcB1 (□) and 3D7 (■) *P. falciparum* strains.

both strains (Table 1). These three classical SERCA inhibitors may not comprise good antimalarial drugs because they are also powerful inhibitors of the mammalian enzyme [18,34] and, consequently, they are expected to be toxic to mammalian cells.

Using the SERCA1a crystallographic structure as a template, Arnou *et al.* [18] analyzed the interactions of these three classical SERCA inhibitors with the homologue residues on PfATP6 based on a model of its structure. Because thapsigargin, CPA and BHQ have different binding sites in the rabbit SERCA1a [35,36], it is expected that the binding sites of these inhibitors also involve different residues of PfATP6 [18]. Based on this assumption, we aimed to search for new inhibitors for PfATP6 among several categories of molecules. In this way, we hoped to increase the probability of finding a PfATP6 inhibitor with good antiplasmodial activity.

Improved expression and purification of PfATP6 for large-scale inhibitor screening

We had to adapt our previously established overexpression and purification procedures to prepare larger amounts of purified, active and concentrated PfATP6. Both expression and purification have been described previously [20], with modifications as indicated in the Experimental procedures. Purification is followed either by immunodetection with anti-PfATP6 serum (Fig. 2A) or by Coomassie Blue staining (Fig. 2B). Using this procedure, detergent-solubilized PfATP6 is fixed on the streptavidin-Sepharose resin as a biotinylated PfATP6-BAD protein (Fig. 2A, lane 1). After cleavage by thrombin for 30 min (Fig. 2A, lane 2) and 60 min (Fig. 2A, lane 3), PfATP6 looses its BAD tag. It is eluted from the resin (Fig. 2A, lane 4) with a small amount of PfATP6-BAD and PfATP6 still retained on the resin (Fig. 2A, lane 5). The concentration of the PfATP6 pool on Centriprep was followed (Fig. 2A, compare lanes 4 and 6). The pattern is similar in both lanes (Fig. 2A, lanes 4 and 6) and there is no significant loss of protein during the concentration step (Fig. 2A, lane 7).

To quantify the concentrated purified PfATP6, it was compared with known amounts of sarcoplasmic reticulum Ca^{2+}-ATPase on a Coomassie Blue stained gel (Fig. 2B). The protein purified by this procedure is rather pure (Fig. 2B, lane 4) and its concentration is estimated to be 1 $mg \cdot mL^{-1}$. This value must be determined to achieve a specific activity of PfATP6. In total, we obtained 1.4 mg of concentrated purified PfATP6 from 6 L of yeast culture and 1.3 g of P3 light membranes.

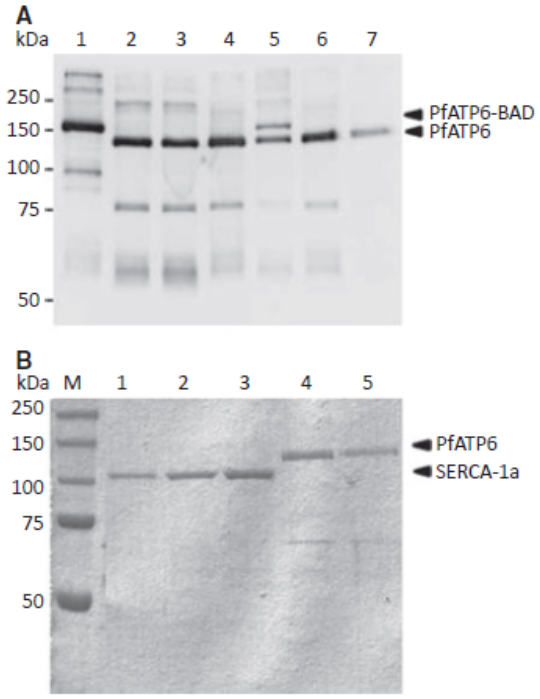

Fig. 2. PfATP6 purification and quantification of purified PfATP6. (A) Western blotting with specific anti-PfATP6 serum. Molecular mass markers are indicated and 0.7 µL of each sample was loaded; streptavidin-Sepharose resin with solubilized light membrane before (lane 1) and after 30 min (lane 2) and 60 min (lane 3) of incubation with thrombin; first elution fraction (lane 4); streptavidin-Sepharose resin after elution (lane 5); eluates after concentration (lane 6); flow-through during concentration (lane 7). We obtained PfATP6 concentrated at a factor 18 and the same volume diluted by a factor 18 was loaded (lane 6). (B) SDS/PAGE and staining with Coomassie Blue. Molecular mass markers (lane M); SERCA1a (SR) loaded at final concentrations of 200 ng (lane 1), 400 ng (lane 2) and 600 ng (lane 3); 5 µL of purified and concentrated PfATP6 was loaded at dilutions of 1 : 20 (lane 4) and 1 : 40 (lane 5) of the concentrated protein.

To verify the enzymatic activity of the purified and concentrated PfATP6 protein, we performed ATPase activity measurements using a classical coupled-enzyme reaction [30,31] (Fig. 3). As described previously [20], ATPase activity of PfATP6 was stabilized by the addition of lipids. Thus, at a octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$) : 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) ratio of 0.2 : 0.05 ($mg \cdot mL^{-1}$), the specific activity at 25 °C of the purified PfATP6 was 0.5 µmol of hydrolyzed $ATP \cdot min^{-1} \cdot mg$ of $PfATP6^{-1}$.

In vitro enzymatic inhibition and antiplasmodial activity of potential PfATP6 inhibitors

A test measuring the inorganic phosphate liberation was established to screen several molecules and choose

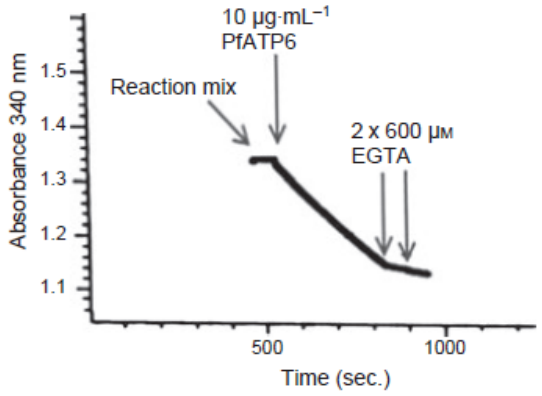

Fig. 3. ATPase activity of purified PfATP6 using a coupled enzyme test. Measurement was performed at 25 °C (pH 7.5) in the presence of $C_{12}E_8$: DOPC (0.2 : 0.05 mg·mL^{-1}) and hydrolytic activity was monitored continuously with a coupled enzyme system by recording NADH oxidation at 340 nm [30,31].

the most promising inhibitors. The screening was undertaken in duplicates on a small library of molecules and the IC_{50} for the inhibition of PfATP6 ATPase activity was determined for 1680 compounds. Twenty of them exhibited a potent inhibitory effect with an IC_{50} of < 10 µM. From these twenty compounds, eight were chosen to test their antiplasmodial activity based on their potent inhibitory effect on PfATP6, as well as their commercial availability. We selected compounds 1, 2, 8, 10, 17, 18, 19 and 20 for this study. The IC_{50} values are listed in Table 2 and the chemical structures are shown in Fig. 4. Their IC_{50} values on purified PfATP6 range between 1 and 9 µM (Table 2) and the best values are for compounds 2, 10, 17 and 18. Thus, these eight molecules comprised a starting point for further studies and were tested for their antiplasmodial activity (Table 2). We observed first that the compounds have a similar inhibitory effect on the growth of either FcB1 or 3D7 strains of *P. falciparum* (in the range 2–250 µM). The antiplasmodial activity of compound 18 is shown in Fig. 1B as an example of the results obtained for the two strains.

Table 2. IC_{50} of the compounds identified to be potent inhibitors of PfATP6 and chosen to be tested on *P. falciparum* cultures. IC_{50} PfATP6: values of the inhibition of the ATPase activity determined on the purified PfATP6; IC_{50} FcB1/IC_{50} 3D7: effect of the identified PfATP6 inhibitors on two *P. falciparum* strains differently resistant to chloroquine (3D7 and FcB1). Values (µM) correspond to at least three biological replicates.

Compound	IC_{50} PfATP6	IC_{50} FcB1	IC_{50} 3D7
1	3.0 ± 2.2	71 ± 25	108 ± 8
2	1.1 ± 0.5	57 ± 15.8	53 ± 19
8	3.1 ± 1.0	88 ± 33	91.5 ± 16
10	1.2 ± 0.6	169 ± 17	241 ± 19
17	1.0 ± 0.6	6.2 ± 1.7	4.9 ± 2.1
18	1.0 ± 0.6	2.3 ± 1.2	3.1 ± 1.2
19	8.5 ± 1.6	14.3 ± 3.6	14.8 ± 7.7
20	7.0 ± 5.4	61 ± 26	62 ± 26

Molecules 17– 20 present a range of action on *P. falciparum* growth *in vitro*; from 2 µM up to ~ 60 µM, with the compound 18 (NSC 95397) presenting the highest antiplasmodial activity (2–3 µM).

Compound 17, also known as Ebselen, presents an IC_{50} for inhibition of purified PfATP6 ATPase activity of ~ 1 µM (Table 2). Hüther *et al.* [37] reported an antiplasmodial activity of ~ 14 µM [37]. Our data lie in the same order of magnitude (~ 4–6 µM) and the difference may be readily explained by the utilization of different *P. falciparum* strains (chloroquine sensitive strain T9-96 and chloroquine resistant strain T9-102) [37]. Ebselen has been extensively studied as an anti-inflammatory drug and has been described to act upon several mammalian enzymes [38].

Compound 18, also known as NSC 95397, appears to be toxic, at least for erythrocytes, because we observed some cell lysis after 48 h of exposure to this molecule at the highest doses tested (> 30 µM) (data not shown). However, it may represent a PfATP6 inhibitor worthy of further exploration, and it is possible that structural modifications will bring a good compromise between toxicity and antiplasmodial activity.

Compound 20, also known as Tyrphostin 47, was previously tested on trypanosome *in vitro* cultures and it was found that nontoxic doses of 1 µM significantly reduced the *in vitro* growth of parasites [39,40], which is a lower concentration than that found when Tyrphostin 47 was tested on *P. falciparum* in the present study (Table 2).

There is no strict correlation between the inhibition of PfATP6 activity and the antiplasmodial activity of these compounds (Table 2). Compounds can inhibit *in vitro* PfATP6 activity and present limited antiplasmodial activity. For example, compound 10 presents a significant inhibition of PfATP6 activity but has a low antiplasmodial effect. One explanation may be that *P. falciparum* possesses mechanisms to extrude drugs and metabolize them, or it may be impermeable to these compounds [41]. Inversely, compounds can inhibit *in vitro* PfATP6 activity and present an antiplasmodial activity for unrelated reasons, such as overall toxicity. To investigate the mammalian cytotoxicity of our compounds, we first determined whether they were inefficient inhibitors of rabbit SERCA1a, which would

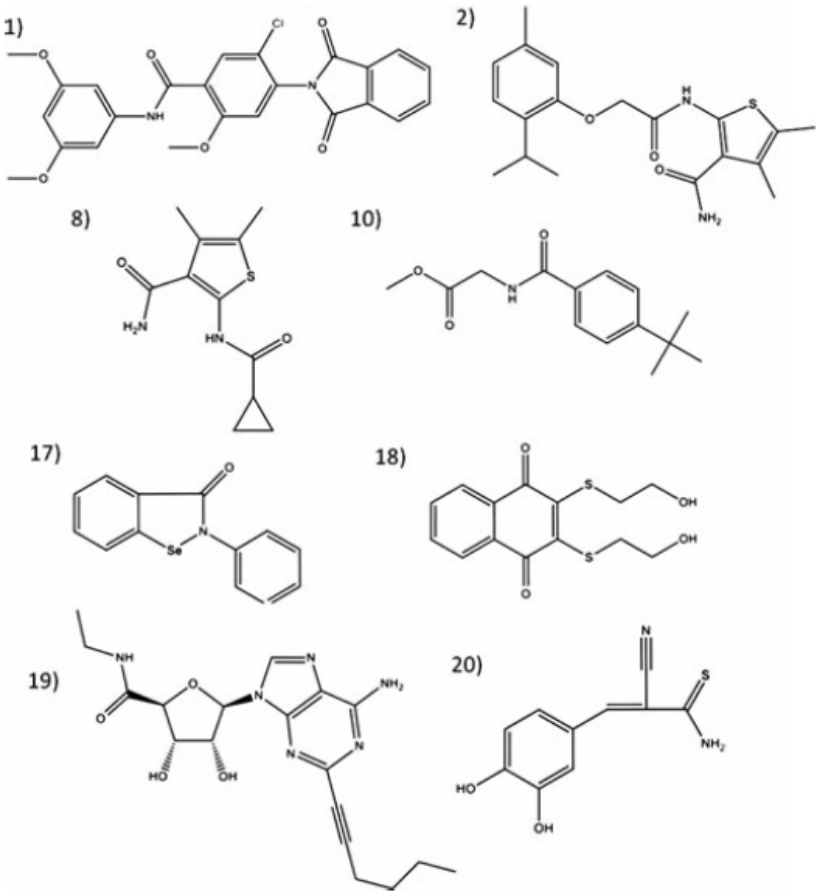

Fig. 4. Structures of the compounds identified to be potent inhibitors of PfATP6.

be a potential advantage in terms of human toxicity. Compound 17 was the only one to significantly inhibit rabbit SERCA1a ATPase activity (IC_{50} of 0.5 μM), whereas the other compounds had little or no effect (50% of inhibition of SERCA1a activity was not reached at a maximum concentration of 20 μM of compound; data not shown). For further development, it will be essential to also exclude cytotoxic effects in mammalian cells.

In summary, Table 2 provides a good starting point for investigating related molecules. Our future studies aim to use these compounds, as well as screen for new compounds issued from our chemical library. The identified inhibitors of PfATP6 display little apparent structural resemblance to already known inhibitors of the SERCA1a Ca^{2+}-ATPase. The binding pocket(s) in PfATP6 for the identified inhibitors are presently unknown and further studies will be needed to identify any possible resemblance to SERCA1a with respect to hit/target interactions. As knowledge of the structure of PfATP6 could ameliorate and accelerate this process, efforts are also being undertaken in this respect.

Concluding comments

There is no optimal solution with respect to the search for new treatments for malaria. Worldwide, researchers have focused their energies in several directions aiming to cover the largest number of possible strategies for discovering novel ways to cure/eradicate malaria. The most commonly employed strategies are: (a) the screening of molecules issued from chemical libraries either in a phenotypic way (i.e. testing compounds upon *in vitro* cultures of *Plasmodium*; the whole cell approach) [8] or (b) searching for inhibitory molecules acting upon the biochemical activity of a potential drug target that comprises an essential enzyme or pathway, ideally specific to the parasite (i.e. a target-based approach) [42].

In the present study, we aimed to establish a collaborative work in a target-based approach, where we could benefit from the knowledge and facilities available to several teams to: (a) screen for molecules that would inhibit PfATP6 activity; (b) select the best inhibitors to subsequently test on *P. falciparum* growth *in vitro*; (c) test the toxicity on mammal cells of the best molecules that

issued from these tests; (d) and, finally, test the efficiency *in vivo* (on a murine model for malaria) of the best inhibitors. So far, the first two objectives have been performed successfully. We are now able to screen for new PfATP6 inhibitors in a large-scale manner, reducing time-consuming issues. This is highly advantageous because we can rapidly eliminate less interesting molecules and focus our efforts on more promising compounds for investigation using a well established *in vitro* antiplasmodial test [43].

One long-term goal is to obtain a very efficient inhibitor against PfATP6, which at the same time is not very efficient for inhibiting mammalian SERCA1a. The most promising compounds should not be toxic to humans, which is an overall stringent requirement. Nevertheless, if they are found to be toxic against human cell lines, it is always possible to consider the pro-drug approach, which has already successfully applied with thapsigargin and cancer cells [10].

Experimental procedures

Chemicals

All chemical products were purchased from Sigma (St Louis, MO, USA) unless specified otherwise. High-activity bovine thrombin was obtained from Calbiochem (San Diego, CA, USA), and the streptavidin-Sepharose High Performance resin was purchased from GE Healthcare (Milwaukee, WI, USA). All products for yeast and bacteria cultures were purchased from Difco (BD Biosciences, Franklin Lakes, NJ, USA). DOPC was obtained from Avanti Polar Lipids (Alabaster, AL, USA). DDM was obtained from Anatrace (Maumee, OH, USA) and $C_{12}E_8$ was purchased from Nikkol Chemical (Tokyo, Japan). Precision protein standards were from Bio-Rad (Hercules, CA, USA). Immobilon-P membranes were obtained from Millipore (Bedford, MA, USA). Phosphoenol pyruvate (catalogue number P3637), L-lactic dehydrogenase solution from bovine heart (catalogue number L1006) and pyruvate kinase preparation type VII from rabbit muscle (catalogue number P7768) were obtained from Sigma. Anti-PfATP6 serum were purchased from Bethyl Laboratories (Montgomery, TX, USA).

Yeast transformation and selection of individual clones

The *Saccharomyces cerevisiae* yeast strain W303.1b/Gal4 (*a, leu2, his3, trp1::TRP1-GAL10-GAL4, ura3, ade2-1, can*^{r}, *cir+*) was the same as described previously [44]. Transformation was performed according to the lithium acetate/single-stranded carrier DNA/polyethylene glycol method [45]. Growth conditions and criteria for expression of the Ca^{2+}-ATPase were carried out as described for the test of individual clones and for the expression on minimal medium [44,46].

Growth of yeast cells and large-scale expression of PfATP6 using a fermentor (Techfors-S Apparatus; INFORS HT, Massy, France)

The method has been described previously [20].

Preparation of light membrane fractions

For membrane fractionation, the procedure was performed as described previously [47], with some modifications. The major change consisted of using a 'pulverisette' for the breaking of yeasts (see below). The yeast suspensions were rapidly thawed in water bath at 20 °C, and one additional volume of Tes buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.6 M sorbitol] with 1 mM phenylmethanesulfonyl fluoride (PMSF) and complete EDTA-free antiprotease cocktail was added before the breaking of the yeast. Cells (200 mL containing 200 g of yeast) were loaded in a grinding bowl agate (500 mL) and one equivalent volume (200 mL) of glass beads (diameter 0.5 mm) was added. Cells were broken using a planetary mill 'pulverisette 6' (Fritsch, Idar-Oberstein, Germany): 3 min at 450 r.p.m., 30-s pause and 3 min at 450 r.p.m. in reverse. The extract was transferred in a cristallisoire and beads were washed three times with 0.5 equivalent volumes (three times in 100 mL) of Tes buffer supplemented with 1 mM PMSF and complete EDTA-free antiprotease cocktail. The resultant crude extract must have a pH in the range 7.0–7.5. The crude extract was centrifuged at 1000 ***g*** for 20 min at 10 °C. The first supernatant S1 was centrifuged at 12 000 ***g*** for 20 min at 10 °C. The second supernatant was removed with great care and was centrifuged at 125 000 ***g*** for 1 h at 4 °C. The pellet P3, containing light membranes, was resuspended in Hepes-sucrose buffer [20 mM Hepes-Tris (pH 7.5), 0.3 M sucrose, 0.1 M $CaCl_2$ supplemented with 1 mM PMSF and complete EDTA-free antiprotease cocktail] (i.e. 0.2 $mL \cdot g^{-1}$ yeast). The membranes were stored at −70 °C until use. The amount of the protein of interest was estimated by western blotting using the appropriate antibody.

Solubilization and batch purification of PfATP6 by streptavidin-Sepharose chromatography

Several of these steps have been modified from previous protocols, such as the washing of the light membrane fraction, the composition of some buffers, the mode of binding of the solubilized solution to the resin and the mode of elution and concentration of the purified proteins. Thus, the light membrane fraction, suspended in the Hepes-sucrose buffer, was washed once to remove contaminant and soluble biotinylated proteins (acetyl-CoA carboxylase, pyruvate carboxylase and Arc1p) by diluting the membranes at 6 $mg \cdot mL^{-1}$ in a buffer containing 50 mM Mops-Tris (pH 7), 0.5 M KCl, 20% glycerol, 1 mM $CaCl_2$, 1 mM

β-mercaptoethanol, 1 mM PMSF and complete EDTA-free antiprotease cocktail. Light membranes were then pelleted at 125 000 ***g*** for 60 min at 10 °C and the supernatant containing soluble proteins was discarded. The pellet was resuspended at a protein concentration of 12 $mg \cdot mL^{-1}$ in the solubilization buffer (same buffer with 0.1 M KCl instead of 0.5 M KCl) with a Potter homogenizer. The same volume of solubilization buffer containing 36 $mg \cdot mL^{-1}$ of DDM was prepared with stirring at 4 °C. Both solutions were mixed and were left for 30 min with stirring at 4 °C, and the solubilizate was clarified by 10–20 up and down with a Potter homogenizer. The clarified solution was left for 30 min with stirring at 4 °C and the nonsolubilized material was pelleted by centrifugation at 125 000 ***g*** for 1 h at 4 °C. All subsequent steps (unless otherwise specified) were then performed at 4 °C. The supernatant after the centrifugation step was mixed with streptavidin Sepharose High Performance resin at a ratio of 4 : 1 (w/v) (previously kept for 15 min at room temperature), using typically 4 mg of PfATP6 per millilitre of resin, and stirred gently overnight at 4 °C. The suspension was then pelleted into 50-mL tubes for 5 min at 500 ***g*** and washed, with ten resin volumes of a 'high-salt' buffer [50 mM Mops-Tris (pH 7), 1 M KCl, 20% glycerol, 1 mM $CaCl_2$, 0.05% DDM) [buffer : resin, 10 : 1 (v/v)], and then with ten resin volumes of a 'low-salt' buffer [50 mM Mops-Tris (pH 7), 100 mM KCl, 20% glycerol, 1 mM $CaCl_2$, 0.05% DDM] [buffer : resin, 10 : 1 (v/v)]. The resin was resuspended in the 'low-salt' buffer [buffer : resin, 1 : 1 (v/v)] and thrombin was added (10 U of thrombin per millilitre of resin) and the mixture was placed on a wheel and gently stirred at room temperature for 30 min, followed by a second addition of thrombin and stirring for another 30 min. To inactivate thrombin, 1 mM PMSF was then added, and the solution of resin was transferred into Handee Centrifuge columns (Perbio Science France SAS, Brebieres, France). The proteolytically cleaved PfATP6 proteins were eluted. A second elution was performed and the eluted fractions containing the Ca^{2+}-ATPase were pooled and concentrated on Centriprep YM30 (Millipore) by successive centrifugations at 1500 ***g*** for 30 min at 4 °C until a volume of 1–2 mL was obtained. The glycerol concentration was increased to 40% before freezing the samples in liquid nitrogen and storage at −70 °C.

Protein estimation, Ca^{2+}-ATPase quantification and rabbit SERCA1a preparation

Protein concentrations were measured by the bicinchoninic acid procedure [48] in the presence of 2% SDS (w/v) with BSA as standard. SERCA1a from rabbit muscle (sarcoplasmic reticulum), used as a standard for protein estimation, was prepared as described previously [49,50]. Ca^{2+}-ATPase quantification was performed on a Coomassie Blue stained gel after SDS/PAGE.

SDS/PAGE and western blotting

For SDS/PAGE, samples were mixed with an equal volume of denaturing buffer and loaded onto Laemmli-type 8% (w/v) polyacrylamide gels [51]. The amounts of proteins or volumes of initial samples loaded in each well are indicated where appropriate. After separation by SDS/PAGE, gels were stained with Coomassie Blue, or proteins were electroblotted onto poly(vinylidene difluoride) Immobilon P membrane [52]. For each gel, molecular mass markers (Precision Protein Standards; Bio-Rad) were loaded. Western blotting was followed by immunodetection with polyclonal anti-PfATP6 serum (dilution 1 : 20 000) generated in goat [20].

ATPase activity measurement using coupled enzymes system

ATPase activity was assayed at 25 °C using a spectrophotometric method as described previously [30,31]. In total, 10 μg of proteins was used in 2 mL of reaction buffer [50 mM TES/Tris (pH 7.5), 0.1 M KCl, 1 mM $MgCl_2$, 0.3 mM NADH, 1 mM phosphoenolpyruvate, 0.1 $mg \cdot mL^{-1}$ lactate dehydrogenase, 0.1 $mg \cdot mL^{-1}$ pyruvate kinase containing 0.06 mM Ca^{2+} and 0.2 : 0.05 $mg \cdot mL^{-1}$ $C_{12}E_8$: DOPC]. The reaction was started by the addition of PfATP6 to the medium and stopped by the addition (twice) of a final concentration of 600 μM EGTA. The difference between the slopes obtained before and after the addition of EGTA is considered to be a result of Ca^{2+}-ATPase activity. Figure 3 shows that there is some residual ATPase activity after the addition of EGTA, which may be a result of PfATP6 not being completely pure and/or a part of PfATP6 possessing a non-Ca^{2+}-dependent activity.

Compound screening

Protein activity was determined by measuring the amount of liberated phosphate (P_i) from the ATP hydrolysis reaction. Some 1 μg of protein per assay and various concentrations of exogenous added compounds were incubated for 10 min in a buffer consisting of 0.1 mM $CaCl_2$, 6 mM $MgCl_2$, 100 mM KCl, 50 mM Tris (pH 7.5), 20% glycerol, 0.2 $mg \cdot mL^{-1}$ $C_{12}E_8$ and 0.05 $mg \cdot mL^{-1}$ DOPC. The reaction was initiated by the addition of Na-ATP to a final concentration of 2.5 mM, followed by incubation for 30 min at 30 °C. The amount of P_i was measured after the addition of STOP-solution [mixture of A: 170.3 μM $C_6H_8O_6$ in 0.5 M HCl; B: 28.3 mM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in Milli-Q H_2O; Millipore] with incubation for 10 min at room temperature followed by the addition of arsenite solution (154 mM $NaAsO_2$, 68 mM $Na_3C_6H_5O_7 \cdot 2H_2O$, 0.3 M CH_3COOH) and incubation for 30 min. D_{860} was measured. A small library of 1680 compounds was screened at a concentration of 16 μM. IC_{50} values were determined

for compounds found to have more than 75% inhibition in the initial screening. Some of these compounds were selected for *in vitro* antiplasmodial testing.

In vitro antiplasmodial tests

The *P. falciparum* strains FcB1 and 3D7 were maintained continuously in culture on human erythrocytes as initially described by Trager and Jensen [32]. Parasite culture medium contained RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY, USA), 25 mM Hepes, 27.5 mM $NaHCO_3$ and 11 mM glucose (pH 7.4), and was supplemented with 7.5% (v/v) compatible heat-inactivated human serum. Human red blood cells were added at a haematocrit of 2% and the parasite cultures were maintained at 37 °C under an atmosphere of 3% CO_2, 6% O_2 and 91% N_2, with daily medium changes. *In vitro* antiplasmodial activities were determined using a modification of the semi-automated microdilution technique of Desjardins *et al.* [33]. Stock solutions of chloroquine diphosphate and tested compounds were prepared in sterile distilled water and dimethylsulfoxide, respectively. Drug solutions were serially diluted with culture medium and added to asynchronous parasite cultures (1% parasitaemia and 1% final haematocrit) on 96-well plates, which were incubated at 37 °C for 24 h in a candle jar system, before the addition of 0.5 μCi of [^{3}H]hypoxanthin (1–5 Ci·mmol^{-1}) per well for an additional 24 h. Parasites were then harvested on filters after a freeze-thawing cycle, and dried filters were submerged in a liquid scintillation mixture (OptiScintHisafe; Perkin Elmer, Boston, MA, USA) and counted in a 1450 Microbeta counter (Wallac; Perkin Elmer). Parasite growth inhibition was determined by comparison of the radioactivity incorporated into the treated wells with that of control wells (containing parasite cultures without drug) from the same plates. The concentrations of drugs that inhibited growth by 50% (IC_{50}) were determined graphically from drug concentration–response curves. Final IC_{50} values for each compound were expressed as the mean ± SD of values determined from independent experiments (biological replicates) [43].

Chloroquine diphosphate (Sigma-Aldrich Chimie SARL, St Quenti Fallavier, France) was used to determine the level of resistance of the FcB1 and the 3D7 strains in the culture and test conditions, and as positive controls of antiplasmodial activities.

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I.3 - Cytotoxicity of the Molecules Tested on P. falciparum In vitro Culture

One of our perspectives to the precedent work was to test the cytotoxicity on mammalian cells, of the compounds tested on *P. falciparum in vitro* culture.

In collaboration with Pr. Isabelle Florent, we established a protocol to identify the cytotoxicity effect of these molecules on Vero cells, reported on CC_{50} [18] values. Vero cells are kidney epithelial cells extracted from an African green monkey (*Chlorocebus* sp.). They are frequently used in cytotoxicity determination experiments. The Vero cells are put in contact with different concentrations of each molecule to test. After 48h of contact with the molecule, a MTT colorimetric assay is performed. This test uses 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), that is a yellow compound when in a water solution. In living cells, it is reduced by a mitochondrial reductase to an insoluble purple formazoan. The formation of these purple crystals is proportional to cell growth, and the absorption can be measured by a spectrophotometer at a certain wavelength between 500 and 600 nm (here we used 570 nm). Using a range of known cell counts, we can easily report the absorbances obtained with cell number and estimate an CC_{50} for cell growth, and hence the cytotoxic effect of the tested molecule.

In Table 9, we present a summary for the IC_{50} values of the PfATP6 inhibitors tested on the purified enzyme from the above article (IC_{50} PfATP6) is represented (David-Bosne et al., 2013); on two laboratory *P. falciparum* strains differently sensitive to chloroquine (3D7 and FcB1) (IC_{50} *P. falciparum*); as well as the complementary cytotoxicity tests on Vero cells (CC_{50} Vero).

Compounds	IC_{50} PfATP6 (µM)	IC_{50} *P. falciparum* (µM)		CC_{50} Vero (µM)	Ratio CC_{50} Vero/IC_{50} *P. falciparum* (µM)	
		3D7	FcB1		3D7	FcB1
1	3 ± 2.2	108 ± 8	71 ± 25	303 ± 52	2.8	4.3
2	1.1 ± 0.5	53 ± 19	57 ± 15.8	92 ± 28	1.7	1.6
8	3.1 ± 1.0	91.5 ± 16	88 ± 33	275 ± 27	3	3.1
10	1.2 ± 0.6	241 ± 19	169 ± 17	500 ± 0	2.1	3
17	1 ± 0.6	4.9 ± 2.1	6.2 ± 1.7	75 ± 27	15.3	12.1
18	1 ± 0.6	3.1 ± 1.2	2.3 ± 1.2	20 ± 41	6.5	8.7
19	8.5 ± 1.6	14.8 ± 7.7	14.3 ± 3.6	500 ± 0	33.8	35.0
20	7 ± 5.4	62 ± 26	61 ± 26	233 ± 4	3.1	3.1

Table 9 - Effect of the tested compounds on purified PfATP6, P. falciparum 3D7 and FcB1 strains in vitro growth and cytotoxicity on mammalian Vero cells.

The effects are expressed in IC_{50} - half maximal inhibitory concentration; and CC_{50} – half maximal cytotoxic concentration.

18 CC_{50} - half maximal cytotoxic concentration – it is the required concentration at which a given tested compound inhibits 50% of a biochemical function or cell/parasite *in vitro* growth.

When looking at the CC_{50} values on Vero cells alone we can't say much about the toxic effect that these molecules have on mammalian cells. For this, it is important to report these values to the IC_{50} obtained on *P. falciparum in vitro* cultures, to be able to know if the quantity required to inhibit 50% of the parasites growth (IC_{50} *P. falciparum*) is much lower than the quantity required to inhibit 50% of the mammalian cells growth (CC_{50} Vero), indicating that a compound is not toxic for cell growth. Medicine for Malaria Venture (MMV) only accepts molecules with low toxicity and high antimalarial effect, this is represented by a ratio between the CC_{50} on mammalian cells reported to the IC_{50} on *Plasmodium sp.* This ratio is required to be above 10, for an effective first hit molecule not to be toxic for humans. In Table *10*, we can see the requirements for the first stage of screening and validation of compounds hits.

Evaluation Level	Test	Consensus
Screening hits	Biological target assay	Biochemical activity at IC_{50} < 3µM
	Cellular assay	Activity against *P. falciparum in vitro* growth (strains 3D7, HB3, DD2, NF54 or W2) at an IC_{50} < 1 µM
Validated Hit	Re-testing	Primary results validated on compounds > 90% pure
	Biological activity	IC_{50} against biological target and whole parasite < 1 µM
	Selectivity	Activity against mammalian enzyme and mammalian cells HepG2 (Hepatocellular carcinoma, human) > 10 – fold selectivity

Table 10 - Medicine for Malaria Venture (MMV) requirements for first stage validated compound hits.
Adapted from http://www.mmv.org/.

Some of our molecules presented a good IC_{50} value (< 3µM) for screening hit on the biological target (PfATP6) but were not very efficient on *P. falciparum* (molecules 1, 2, 8, 10, 19 and 20). If we look at the MMV criteria for a validated target (Table *10*), molecules 17 and 18 present an IC_{50} on the target PfATP6 ≤ 1µM, and a quite low IC_{50} on *P. falciparum* 3D7 strain (3 – 5 µM). Although this value is higher than the required by MMV for a validated compound, it is rather interesting starting point. Especially for compound 17, that has a fairly low toxicity, with at least a 10-fold selectivity, although compound 18 is not that far from reaching the threshold. These are promising molecules that could be rearranged chemically to ameliorate the affinity to PfATP6, the antimalarial activity and the cytotoxicity (Table 9).

Compound 19, although it does not enter the MMV criteria for the IC_{50} values on the biological target neither on *P. falciparum* 3D7 (Table *10*), presents a relatively low toxicity. This could be chemically attractive to exploit by trying to improve the affinity to PfATP6 as well as the antimalarial activity, to enter MMV criteria (Table *10*).

It is important to indicate that these molecules have been tested on the mammalian target SERCA1a, and did not significantly inhibit its biochemical activity (see some of them Figure 31). This is very important and indicates that these molecules are specific inhibitors of PfATP6, and might have low toxicity, which is verified for some compounds by high IC_{50} on Vero cells (Table 9).

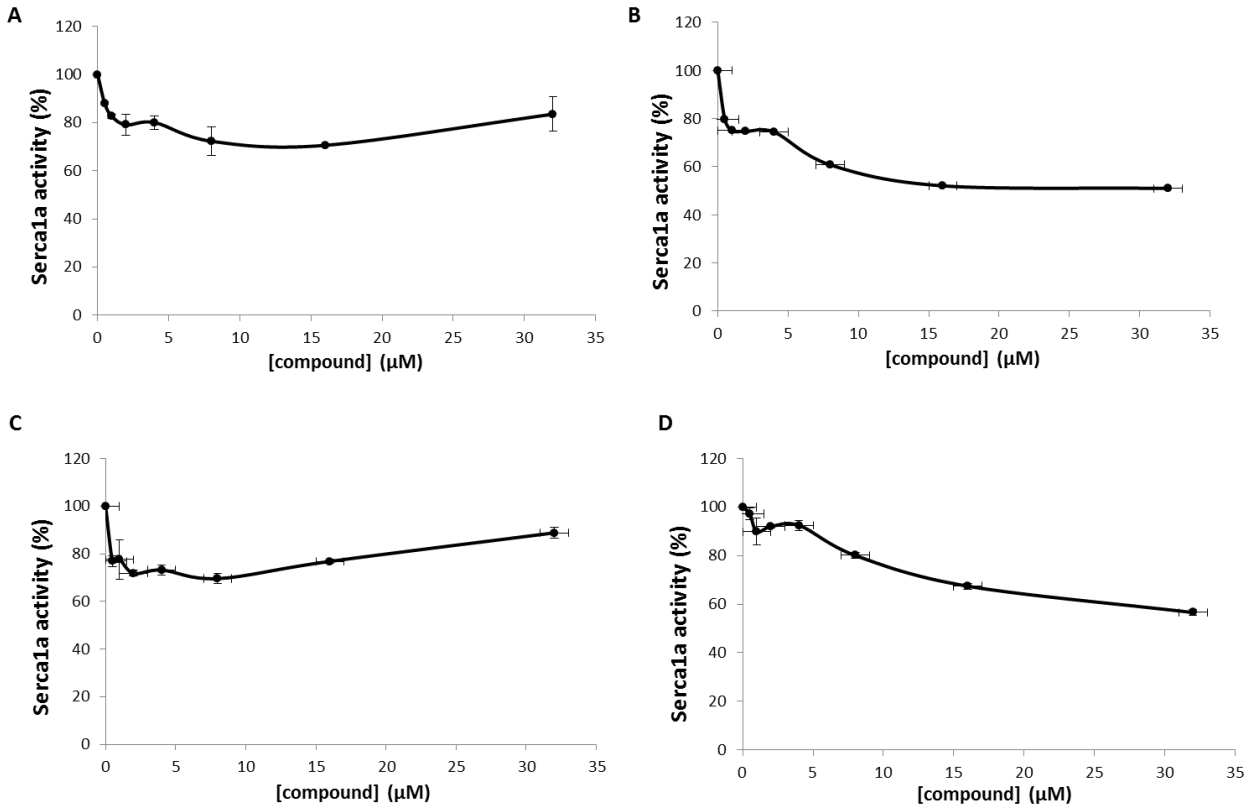


Figure 31- Effect of Pcovery compounds on SERCA1a from rabbit muscle.

A) *compound 1;* ***B)*** *compound 2;* ***C)*** *compound 8;* ***D)*** *compound 10 (compounds are referenced in David-Bosne et al 2013).*

The inhibitors found 17, 18 and 19 are promising structures to explore and improve to be proposed to MMV as future antimalarials. Compound 17 would need to be improved for its antimalarial activity to go under 1 µM. Compound 18 has to be modified for reducing cytotoxicity on mammalian cells but also for an antimalarial activity < 1 µM. Compound 19 has a longer way to go to reach MMV criteria on the IC_{50} values on the biological target and on *P. falciparum* 3D7, although it is already a compound with low cytotoxic effect. For this it would be interesting to call for the expertise of chemists and start new collaborations.

Although these tests are preliminary, they indicate good potential molecules to be chemically exploited. It would be important to test these molecules on other mammalian cells such as HepG2 (Hepatocellular carcinoma, human), but also *in vivo,* on a malaria rodent model.

I.4 – Establishment of an Activity Measurement Protocol in 96-well Microplate and Inhibitor Testing

I.4.1 – Activity measurement in a 96 well microplate

After the publication of the above article (David-Bosne et al., 2013) we have been contacted by an Italian team that was interested in our expertise in purifying PfATP6 and inhibitors screening. A collaboration was established for testing some of their molecules.

In the past, we routinely measured PfATP6 Ca^{2+}-dependent ATPase activity in a 2 ml cell of a spectrophotometer, using a coupled enzyme test that detected the reduction of NADH, directly linked to the consumption of ATP (Figure 32). As we used a final concentration of protein of 10 µg/ml, this experiment consumed a lot of protein (Cardi et al., 2010b). To test new molecules we needed to develop a test that consumed less protein. For this we established a protocol for ATPase activity measurement in 96 well microplates. The protocol is based on a colorimetric test that measures ATP consumption by detecting the inorganic phosphate (Pi) released. A colorimetric solution with ammonium molybdate and ascorbic acid is used. Ammonium molybdate reacts with Pi and is reduced by ascorbic acid, in a pH 5 environment. The initial colorimetric solution is yellow and with the reduction of the phosphomolybdate, in function of the quantity of Pi present in the medium, the solution changes color to blue. The blue color gets more intense proportionally to the quantity of Pi present, and can be measured at 850nm (Drueckes et al., 1995).

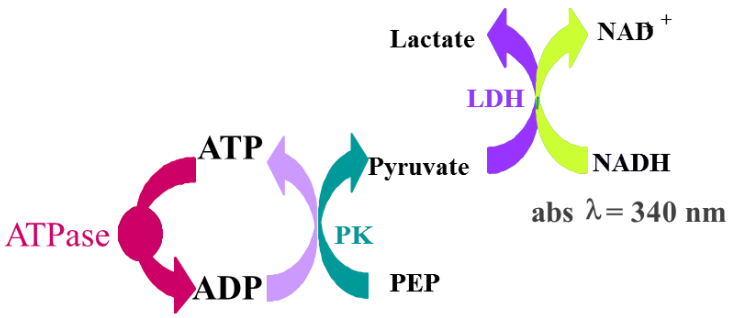


Figure 32 - Schematic representation of coupled enzyme ATPase activity measurement.
LDH – Lactate DesHydrogenase; NAD - Nicotinamide Adenine Dinucleotide; PEP – PhosphoEnol Pyruvate; PK – Pyruvate Kinase.

The protocol was first optimized with sarcoplasmic reticulum from rabbit muscle (SR) containing mainly SERCA1a (Figure 33 A). In presence of calcium ionophore A23187 and calcium at 30ºC, the measured activity of SR is around 2.5 µmol of Pi released.min^{-1}.mg^{-1} of SR (Figure 33 A, blue line). This value is lower than what is obtained by the coupled enzyme test, but an inhibition of the activity

(here demonstrated in presence of calcium and its chelator EGTA, Figure 33 A green line) can clearly be visualized, which is enough to determine the effect of specific inhibitors.

This protocol was then optimized for purified PfATP6 in 96 wells microplates. In Figure 33 B we can observe PfATP6 activity in presence and absence of calcium (last in presence of EGTA, Figure 33 B respectively blue and green line). PfATP6 presents a highest activity in presence of detergent and lipids ($C_{12}E_8$ and DOPC, Figure 33 B blue line) rather than only in detergent ($C_{12}E_8$, Figure 33 B pink line) (Cardi et al., 2010b). PfATP6, behaves in the same way as previously described (Cardi, 2009), it needs lipids to be active and stable. Also, Ca^{2+} ATPases when detergent-solubilized, can present an altered ATPase activity (Lund et al., 1989). In these conditions, we are able to measure an activity of 0.35 µmol of Pi released.min^{-1}.mg^{-1} of PfATP6. This activity is less then that obtained previously with the classical ATPase coupled enzyme activity test (Arnou et al., 2011; Cardi et al., 2010b; David-Bosne et al., 2013). Morever, the activity measurement presented Figure 33 B clearly indicates that the enzyme is less active between 30 and 60 minutes of incubation at 30°C.

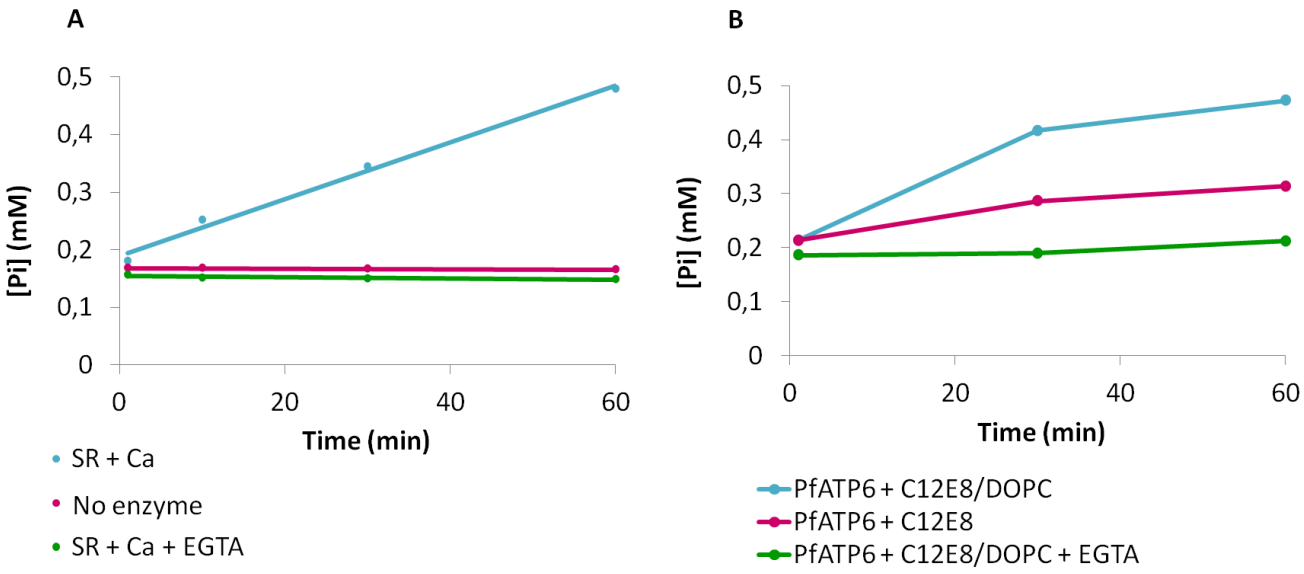
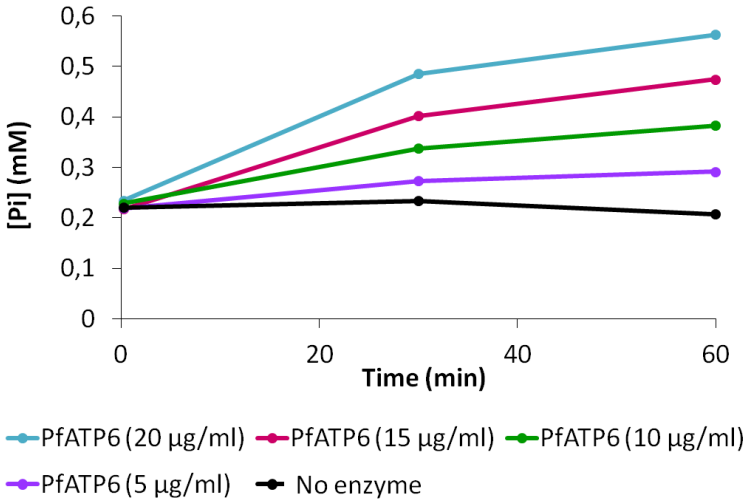


Figure 33 - SR and PfATP6 ATPase activities measurement by Pi releasing.

A) *SR Ca^{2+} dependent ATPase activity. Blue line- SR activity in the presence of A23187 and calcium; pink line – no enzyme; green line – SR in the presence of A23187 + Calcium + EGTA. Time points were linked with a linear trendline.* ***B)*** *PfATP6 Ca^{2+} dependent ATPase activity. Blue line- PfATP6 in $C_{12}E_8$/DOPC and calcium; pink line – PfATP6 in the presence of $C_{12}E_8$ and calcium; green line – PfATP6 in $C_{12}E_8$/DOPC + calcium + EGTA. Experience undertaken at 30ºC, and ratio of $C_{12}E_8$/DOPC of 2:0.5 mg/ml. Three time points were collected (0, 30 and 60 min). SR – Sarcoplasmic reticulum containing rabbit SERCA1a; [Pi] – concentration of inorganic phosphate released.*

A



B

PfATP6 20 µg/ml - 0.42 µmol of Pi released.min^{-1}. mg^{-1} of PfATP6
PfATP6 15 µg/ml - 0.41 µmol of Pi released.min^{-1}. mg^{-1} of PfATP6
PfATP6 10 µg/ml - 0.37 µmol of Pi released.min^{-1}. mg^{-1} of PfATP6
PfATP6 5 µg/m - 0.36 µmol of Pi released.min^{-1}. mg^{-1} of PfATP6

Figure 34 - Determination of PfATP6 optimal concentration for ATPase activity measurement test by Pi releasing.

A) *Blue – 20 µg/ml PfATP6; Red – 15 µg/ml PfATP6; Green – 10 µg/ml PfATP6; Purple – 5 µg/ml PfATP6; Black – no enzyme. Experience undertaken at 30ºC, and ratio of $C_{12}E_8$/DOPC of 2:0.5 mg/ml. Three time points were collected (0, 30 and 60 min). B) PfATP6 specific Ca^{2+}-dependent ATPase activity measurement in function of purified protein concentration. [Pi] – Concentration of inorganic phosphate released.*

In these conditions, we tested various amounts of PfATP6 (Figure 34). It appears that 15 µg/ml is sufficient to obtain a specific activity of 0.4 µmol of Pi released.min^{-1}.mg^{-1} of PfATP6. With 15 µg/ml of enzyme, we tried to reduce the time of incubation to 15 minutes (from 0- 15 min the measured ATPase activity remained linear). By this way, we obtained a higher specific activity of 0.6 µmol of Pi released.min^{-1}.mg^{-1} of PfATP6, which is similar to the activity measured previously by using the classical coupled enzyme ATPase activity test (David-Bosne et al., 2013).

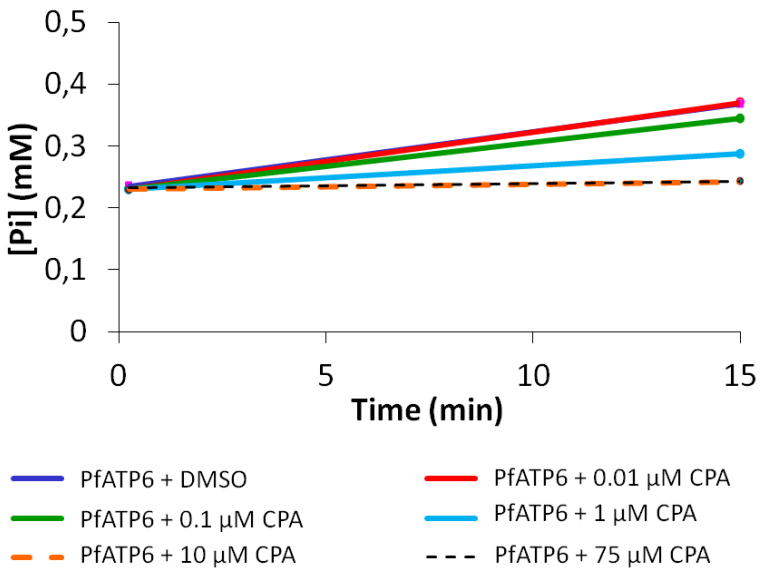
I.4.2. – Inhibitors testing on purified PfATP6

After adaptation of the protocol of Ca^{2+}-dependent ATPase activity measurement by detection of released Pi with purified PfATP6, inhibitors could be tested on the purified enzyme. First the determination of the effect of DMSO on PfATP6 specific activity was performed. We tested serial dilutions of DMSO according to the concentrations that will be applied when the compounds will be tested (2.5% DMSO). For this, we first preincubated the enzyme in the presence of DMSO before the incubation with ATP. We observed the same loss of activity as compared with the assay without DMSO preincubation. This is consistent with the protein instability at 30°C. Consequently, we suppressed this preincubation. Moreover, we were able to exclude a major effect of DMSO on this activity (data not shown). With this we can determine a specific effect of the compound on PfATP6 activity.

In a first experiment we aimed to perform a test of inhibition of PfATP6 activity using classical inhibitors of SERCA, and compare the published results (Arnou et al., 2011; Cardi et al., 2010a) with the ones obtained with this test. For this we tested several concentrations of cyclopiazonic acid (CPA) on purified PfATP6 (Figure 35 A). We could draw a dose-response curve (Figure 35 C) and determine IC_{50} concentrations for the tested compound (indicated by the red horizontal line in Figure 35 C). For CPA the concentration needed to inhibit 50% (IC_{50}) of PfATP6 activity in these conditions is of ~ 0.4 µM, and at ~10 µM we can inhibit more than 90% of PfATP6 activity.

After the optimization of the protocol for PfATP6 inhibitors testing, we have finally tested the compounds sent by the Italian team (Figure 37). They constructed a hybrid antimalarial molecule that combines a 4-aminoquinoline pharmacophore of chloroquine with that of a clotrimazole-based compound, an antifungicide-based compound that was identified to be a potent antiplasmodial compound targeting the hemoperoxydase of *P. falciparum* (Trivedi et al., 2005). These hybrid compounds (Gemma et al., 2009) were optimized and tested for toxicity and mutagenicity as well as preliminary pharmacokinetic analysis and administration assays against chloroquine-resistant parasites (Gemma et al., 2012). One of these hybrid compounds was found to be a potent antimalarial (4b – Figure 36). These molecules are potent antimalarials that imitate the mechanism of Saquinavir, an antiretroviral drug that is known to act synergistically with chloroquine against choloriquine resistant parasites (Martin et al., 2012). Some of these hybrid molecules were described to inhibit SERCA1a (Bartolommei et al., 2011) but also Chloroquine (CQ) transport in CQ_resistant PfCRT, when this protein is expressed in *Xenopus laevis* oocytes (Gemma et al., 2012). For this reason, they aimed to test two of them and 7 new compounds on PfATP6 in order to determine if this protein is the target.

A



B

2.5 % DMSO – 0.6 µmol of Pi released.min $^{-1}$.mg^{-1} of PfATP6
0.01 µM CPA – 0.63 µmol of Pi released.min $^{-1}$.mg^{-1} of PfATP6
10 µM CPA – 0.05 µmol of Pi released.min $^{-1}$.mg^{-1} of PfATP6

C

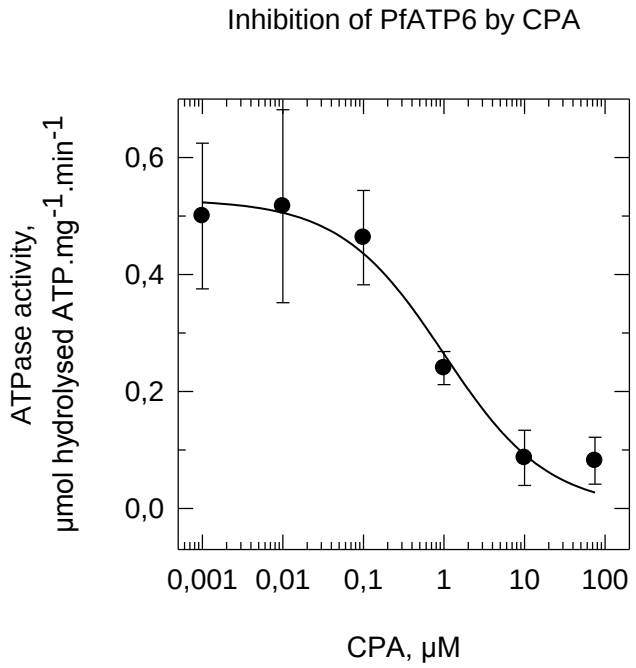
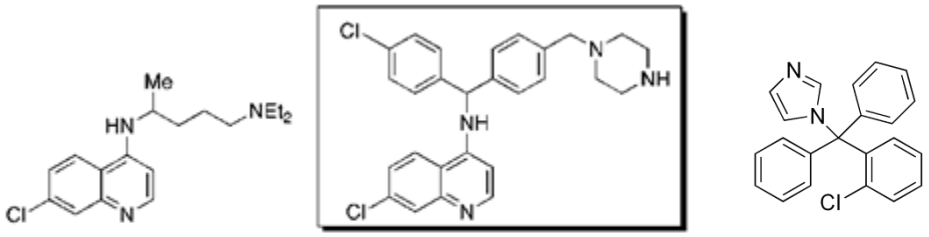


Figure 35 – Effect of CPA on PfATP6 activity.

A) *Effect of several concentrations of CPA on PfATP6 activity. dark blue – PfATP6 + DMSO; red – PfATP6 + 0.01 µM CPA; dark green – PfATP6 + 0.1 µM CPA; light blue – PfATP6 + 1 µM CPA; dashed orange– PfATP6 + 10 µM CPA; dashed black – PfATP6 + 75 µM CPA;* ***B)*** *Specific activity of PfATP6 with different concentration CPA (0.01 and 10 µM) and DMSO (2.5%);* ***C)*** *Effect of serial concentrations of CPA on the activity of purified PfATP6 (µmol of Pi released.min^{-1}.mg^{-1} of PfATP6). Experience undertaken for 15 minutes at 30ºC, and ratio of $C_{12}E_8$/DOPC of 2:0.5 mg/ml. CPA – cyclopiazonic acid; [Pi] – concentration of inorganic phosphate released.*



Chloroquine
Substrate of PfCRT-CQR

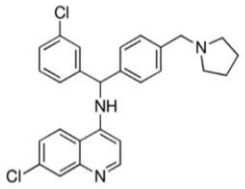
4b
Antimalarial against PfCRT-CQR

Clotrimazole

Figure 36 - Hybrid antimalarial (4b) based on chloroquine and clotrimazole structures.
PfCRT-CQR – P. falciparum Chloroquine resistant transporter (PfCRT) in chloroquine resistant parasites (CQR) (Gemma et al 2012).

Compounds	NF1058	NF1442	NF1255	NF1440	NF1952	NF2003	NF2059	NF2067	NF2068
IC_{50} (µM) PfATP6	> 100	ND	100-200	200-600	> 100	ND	85-100	75-200	100-200
IC_{50} (µM) SERCA1a (Bartolomei et al 2011)	8 ± 2	1.3 ± 0.2							

NF1058



NF1442

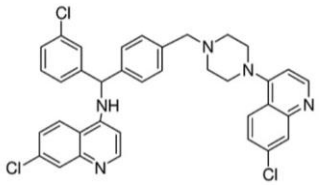


Figure 37 - Effect of a 4-aminoquinoline / clotrimazole-based hybrid compounds on purified PfATP6 and on rabbit sarcoplasmic SERCA1a.
The effect of each compound on purified PfATP6 is expressed in IC_{50} (µM) and were determined by Ca^{2+} - dependent ATPase activity measured by Pi liberation in a 96 well plate. The results for sarcoplasmic reticulum SERCA1a were determined by Bartolomei et al., (2011). The chemical structures correspond to the tested compounds NF1058 and NF1442 (Bartolommei et al., 2011). ND – non determined.

We aimed by this to verify if PfATP6 would be the direct target of these compounds. Unfortunately these compounds were poorly efficient and an IC_{50} was hard to determine (see Figure 37). These compounds did not seem to target directly PfATP6, even though the two compounds inhibited SERCA1a (Bartolommei et al., 2011). This specific preparation of purified PfATP6 used was verified and confirmed to be active and inhibited by the known potent specific inhibitor (CPA – (Arnou et al., 2011; Cardi et al., 2010b) (Figure 34 B et Figure 35 A), excluding a potential problem with the protein or the activity measurement protocol.

Although we did not find significant results with these compounds, we were able to optimize a screening procedure for new PfATP6 inhibitors identification that can be performed in our laboratory conditions.

I.5 – Improvement of PfATP6 Expression and Purification Protocol

To produce our target membrane proteins we classically use yeast (*Saccharomyces cerevisiae*) as a heterologous expression system. To achieve this, the gene of the protein to express, in this case a yeast codon optimized *pfatp6* gene, was inserted in the yeast expression vector pYeDp60 (Pompon et al., 1996), under the control of a galactose inducible promoter and fused to a BAD [19] domain (Figure 38). The codon optimization for the expression of *Plasmodium* genes in yeast was revealed important in the past, because mRNA from these genes are often prematurely truncated when expressed in this heterologous system (Sibley et al., 1997). Yeast that have positively been transformed with this vector construction, are identified by a previous culture in minimum medium. The chosen clone is then cultured and the expression of PfATP6 is triggered by the addition of galactose. During the expression phase, PfATP6-BAD is endogenously biotinylated (Figure 39, Figure 40). The expression of PfATP6 can be detected either by a specific antibody (a polyclonal antibody generated in goat targeting the N-domain position 574-588, provided by S. Krishna); or by an avidin peroxidase probe that specifically recognizes biotinylated proteins (Figure 39). Other than PfATP6, there are proteins that are naturally biotinylated in yeast: Acetyl CoA Carboxylase (250 kDa), Pyruvate carboxylase (120 kDa), and Arc1p (45 kDa) (see Figure 39). The BAD domain enables also the selection of well folded proteins, as it is required that this domain is well folded to accept a biotin. Indeed, during protein synthesis and folding, PfATP6 will follow the same process as BAD (Cardi et al., 2010a; Jidenko et al., 2006).

[19] BAD – Biotin Acceptor Domain. *BAD domains are used because they are reputed to select properly folded proteins, even though the final yield of the purification is inferior when compared to other methodologies used with other tags, the proteins purified are well folded and active. This was previously reported for proteins produced in Pichia pastoris and purified with N^{2+} - nitrilotriacetic* (Tan et al., 2006; Zhang et al., 2002).

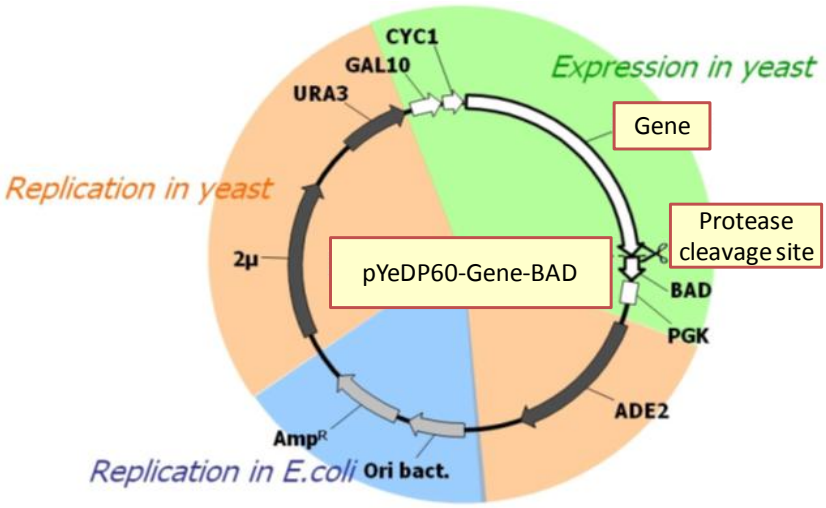


Figure 38 - Yeast expression vector (pYeDp60) construction with gene of interest.

Cloning and amplification in Escherichia coli (light grey): Ori bact - bacterial replication origin; Amp ^{R} - gene coding for β -lactamase to allow resistance to ampicillin (selection marker). Amplification in yeast (dark grey): ADE2 - auxotrophy selection marker for adenine; URA3 - auxotrophy selection marker for uracil; 2µ - yeast replication origin. Expression (white): GAL10-CYC1 - fusion promoter of the inducible part of GAL10 and RNA polymerase binding part of CYC1; PGK - phosphoglycerate kinase terminator sequence; Gene coding sequence; Protease cleavage site - sequence coding for a protease cleavage site; BAD - biotin acceptor domain. The Gene, protease cleavage, and BAD sequences were cloned in the same coding frame to allow expression of the fusion protein-BAD.

After expression, we break the yeast membranes and we proceed to membrane separations. The crude extract (CE) corresponds to the broken yeast after milling in the *Pulverisette*. At this stage we have all membrane fractions that will then be separated by differential centrifugation (see Figure 39). After a first low speed centrifugation of the CE, the pellet (P1) is discarded and the supernatant (S1) is centrifuged at an intermediate speed to obtain a P2 membrane pellet and a S2 supernatant. The analysis of these membrane fractions gives us the indication of how much PfATP6 is either misfolded or addressed to another membrane compartment than the endoplasmic reticulum, and is thus lost in the P2 membranes. These membrane fractions P2 usually contain a large amount of PfATP6. The purification of an expressed protein from these membranes is very hard to achieve as the solubilization is difficult. Furthermore the specific activity of the purified protein is low (Cedric Montigny, personal comunication). These results were confirmed with SERCA1a-BAD produced in yeast by Marie Jidenko (Jidenko et al., 2006), suggesting that the proteins addressed to these non-native compartments might be either misfolded or just less active in this membrane environment (see Figure 39).

The P3 membranes are obtained after high speed centrifugation of the supernatant (S2), from the intermediate centrifugation that originated P2 membranes. These membranes are enriched with microsomes and contain the largest fraction of well folded PfATP6 that is addressed to its native compartment (endoplasmic reticulum) (see Figure 39). PfATP6 is present at 2% of the total membrane proteins (data not shown).

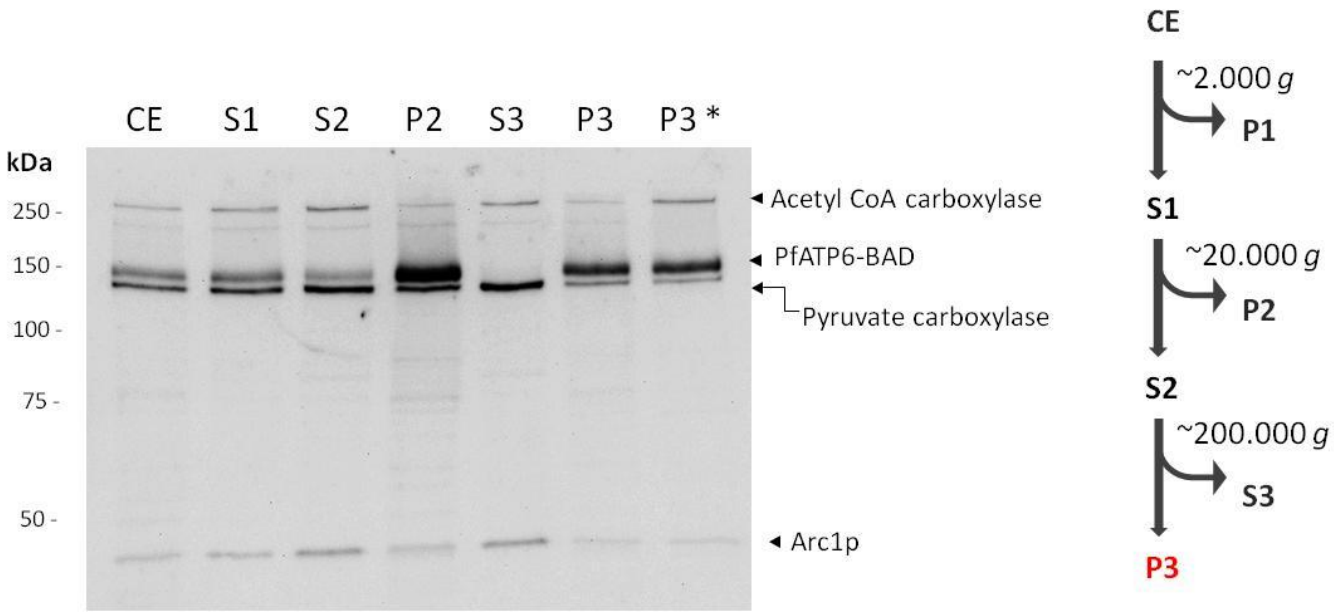


Figure 39 - Expression of PfATP6 in yeast.

Following of membrane preparation : CE – crude extract; S1 – supernatant of the low speed centrifugation; S2 – supernatant of the intermediate speed centrifugation; P2 – pellet of the intermediate speed centrifugation; S3 – supernatant of the high speed centrifugation; P3 – pellet after high speed centrifugation; P3 - P3 from a previous membrane preparation. Detected proteins: Acetyl CoA Carboxylase (250 kDa), Pyruvate carboxylase (120 kDa), Arc1p (45kDa), PfATP6-BAD (148 kDa). 1µg of total protein is deposited; detection was done with an avidin peroxidase probe.*

The solubilization of the membranes is done with mild detergent; for membranes expressing PfATP6 we used DoDecyl Maltoside (DDM) (Figure 40). According to D. Cardi, ~30% of PfATP6 is biotinylated and PfATP6 is present at 2% of the total membrane proteins in P3 fractions (Cardi et al., 2010b). The biotinylated proteins are then isolated by specific binding to a streptavidin – Sepharose resin. Batch purification is achieved by separation of the BAD domain from PfATP6 by protease cleavage (thrombin was first used for PfATP6). PfATP6 is then eluted, collected and concentrated on a Centricon® column by size exclusion filter (Figure 40).

In this section we aimed to optimize the expression protocol of PfATP6 in order to produce a larger amount of PfATP6. The obtained results are discussed below.

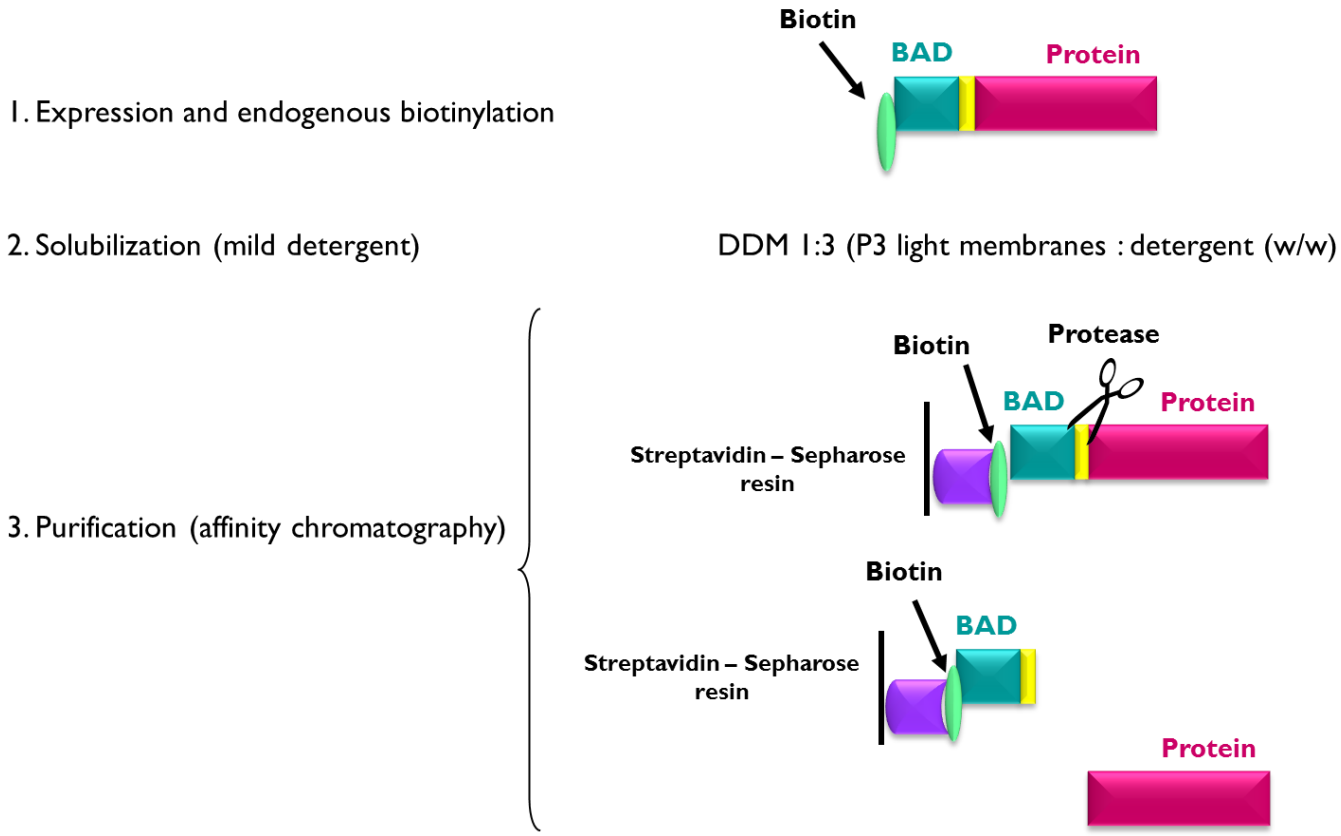


Figure 40 - Schematic representation of PfATP6 purification procedure by purification using the BAD domain by streptavidin-Sepharose chromatography.

I.5.1 – Improvement of PfATP6 expression protocol

The protocol for membrane preparation and purification of PfATP6 was adapted from Cardi et al., 2010b; Jidenko et al., 2006, for large scale production. It is discussed in the article of the present chapter: *I.2 - Article - Antimalarial screening via large-scale purification of Plasmodium falciparum Ca^{2+}-ATPase 6 and in vitro studies*. Posterior to the modification of this procedure, we aimed to verify if the procedure was optimized at all steps, or if we could somehow increase the final yield of the purification. As we had already improved the membrane preparation protocol (David-Bosne et al., 2013) we focused on the expression and the purification of PfATP6. Indeed, we have recently purchased a unit composed with two one-liter fermentors and by using them, it could be easier to tests various conditions. The first step was to verify if the PfATP6 expressions obtained in the 20-liters and in the 1-liter are similar.

The expression of PfATP6 was greatly improved when the culture method was switched from Fernbach flasks to a fermentor. This was mostly because yeast could be grown at higher density and

in more homogenous environment and because, for SERCA1a and for PfATP6, it was observed that a higher amount of these proteins was recovered in P3 membrane fractions (multiplied by 5 for SERCA1a and by 2 for PfATP6). The fermentor also enables the following of a series of parameters such as: dissolved dioxygen, pH, temperature, stirring, and optical density reflecting yeast growth (see Figure 56 of Material and Methods section). Yeast can grow under fermentative or respiration metabolism, they are facultative aerobic cells. When yeast are inoculated into a rich medium, they grow in aerobic conditions using glucose as their carbon source, producing ethanol by fermentation and consuming oxygen. They are in an exponential growth phase. Once glucose is entirely consumed from the medium, the growth rate decreases and the yeast switch to a respiration metabolism, consuming the ethanol produced without oxygen consumption. The growth rate is slowed down by regulating the amount of oxygen in the medium, because when yeast is in anaerobiosis, the growth rate is slowed down and an extended reticulum network is synthethized (Damsky, 1976). The induction of PfATP6 expression is then achieved by adding 2% of galactose and by lowering the temperature from 28ºC (used for growth phase) to 18ºC. The temperature is lowered to 18ºC because it was observed for SERCA1a that proportionally, the quantity of protein is higher in P3 fractions under these conditions, than at 28ºC (Lenoir et al., 2002). And also because lowering the temperature enables yeast growth to slow down, which promotes membrane synthesis for further cell division.

We started by analyzing the expression of PfATP6 in yeast by performing a kinetic of the expression phase. After the first galactose induction, we followed the expression by taking a sample at several time points: 0, 2.5, 5, 13, 15.5, 18, 21 and 24 hours after induction, as described in Figure 41. Classically, a first galactose induction is done after 32 hours post-inoculation and a second galactose induction is performed after 45 hours post-inoculation. In the classical protocol (established in Cardi et al., 2010b), the culture is stopped and yeast are harvested after 50 hours of culture (post-inoculation) but here we decided to go further to see if PfATP6 expression levels would increase.

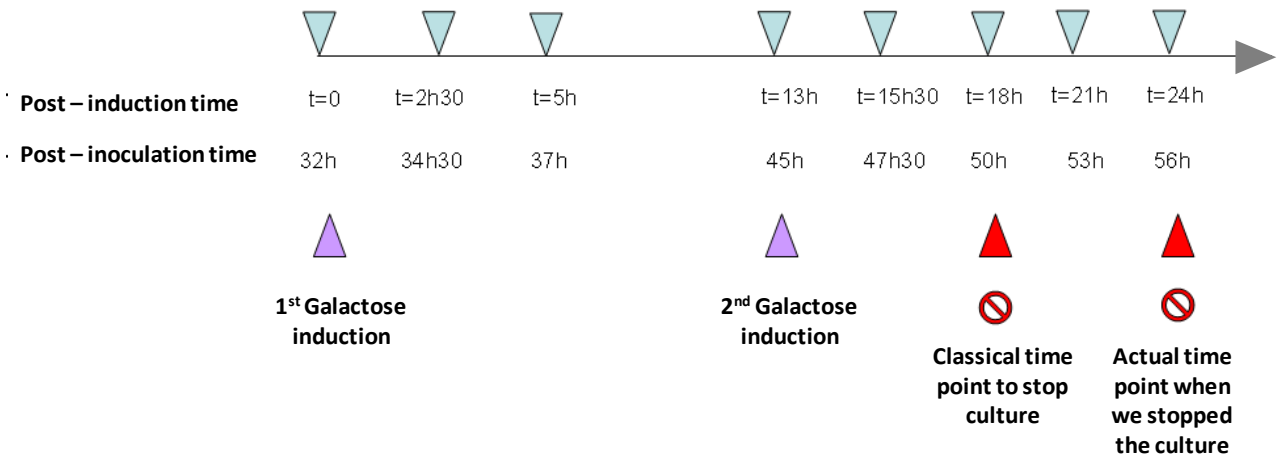


Figure 41 - Schematic representation of the protocol performed for the following of the expression of PfATP6.

We performed membrane preparation for each time point collected and compared the expression levels by western blot (Figure 42 and Figure 43). We can see the differences in PfATP6 quantities through the membrane fractions obtained (crude extract, P2 membranes, P3 light membranes). By analyzing both Figure 42 and Figure 43 (respectively, avidin peroxidase and anti-PfATP6 detection), we can see that PfATP6 begins to express after 5h post first induction (Figure 42 B and Figure 43 B). Only after 13h after the first expression induction, PfATP6 expresses at high levels and is present in microsomes (P3 membrane fractions) (Figure 42 C and Figure 43 C). This also corresponds to the second galactose induction. We here confirm that in the classical expression protocol, the second induction was established at a critical time, corresponding to the starting phase of expression of PfATP6. Hence, the second induction will boost PfATP6 expression, and hence was established at a good time point.

After 18h post first induction the expression of PfATP6 in microsomes remains constant (Figure 42 C and Figure 43 C). With this we also show that it was well established to be the time point to stop the culture and harvest yeast. There is perhaps a slight increase in PfATP6 expression in the P2 membranes (Figure 42 B), but this would only indicate that the post-18h expressed PfATP6 proteins would be misfolded and no more addressed to the right membrane compartment. So a more extended expression time would not be beneficial.

With these experiments we are able to conclude that the expression protocol was applicable for our small fermentors. Both the second induction and the yeast harvesting correspond to important key time points in the expression of PfATP6. It was shown by D. Cardi during her PhD (Cardi, 2009) that a higher expression of PfATP6 resulted in a lower percentage of biotinylation, which suggested that the amount of biotin is limitant, and a supply of this molecule can be considered, although it has been previously attempted with no clear improvement on the biotynilation of PfATP6 (Cardi, 2009). Nevertheless, this addition in a 20-liters fermentor may be very expensive and we are not sure that an increase of biotinylation is a good point. A limited biotinylation may also constitute a control quality for well folded proteins. In the other hand, it seems that PfATP6-BAD goes first into P3 membranes, and PfATP6-BAD stores in P2 membranes only after saturation of the P3 membranes (Cardi, 2009). The same was observed with SERCA1a-His (obtained by P. Falson and G. Lenoir). Maybe if yeast are grown longer in a fermentative exponential growth stage by supplying with more glucose, we could hope to harvest more yeast before too much PfATP6 gets stored into P2 membranes.

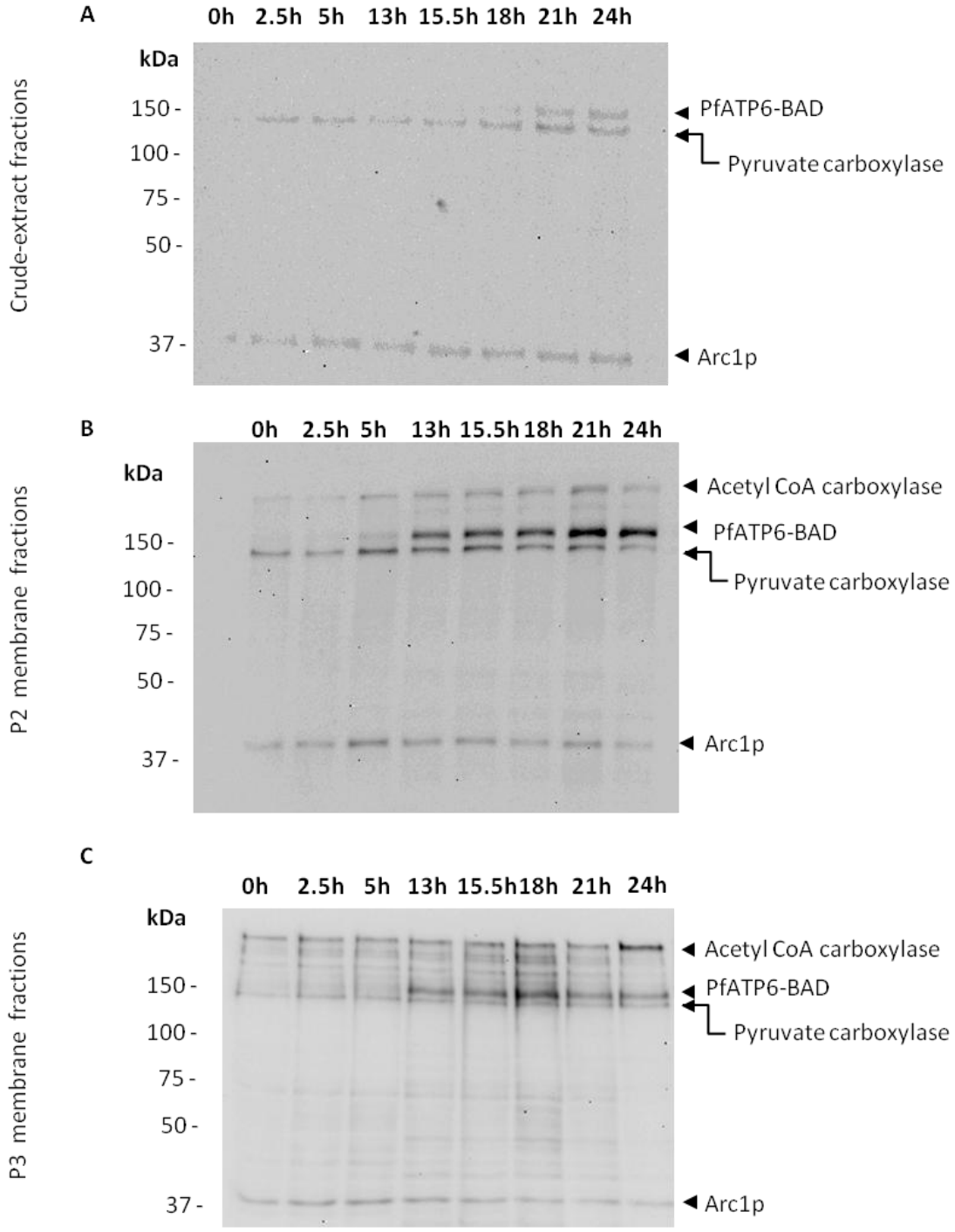


Figure 42 - kinetic of the expression of PfATP6 in yeast. Western Blot revealed with an avidin peroxidase probe (Biotinylated proteins).

*Fractions of the membrane preparation at each time point: **A** – Crude extract (CE); **B** – membranes from the intermediate speed centrifugation (P 2); **C** – Light membrane fractions from the high speed centrifugation (P3). The time points correspond to hours after the first induction, see Figure 41. Detected proteins: Acetyl CoA Carboxylase (250 kDa), Pyruvate carboxylase (120 kDa), Arc1p (45kDa), PfATP6-BAD (148 kDa). 1µg of total protein is deposited. It is important to note that the protein quantity between each western blot is not comparable because these are independent experiments and the pictures were taken with different exposition times.*

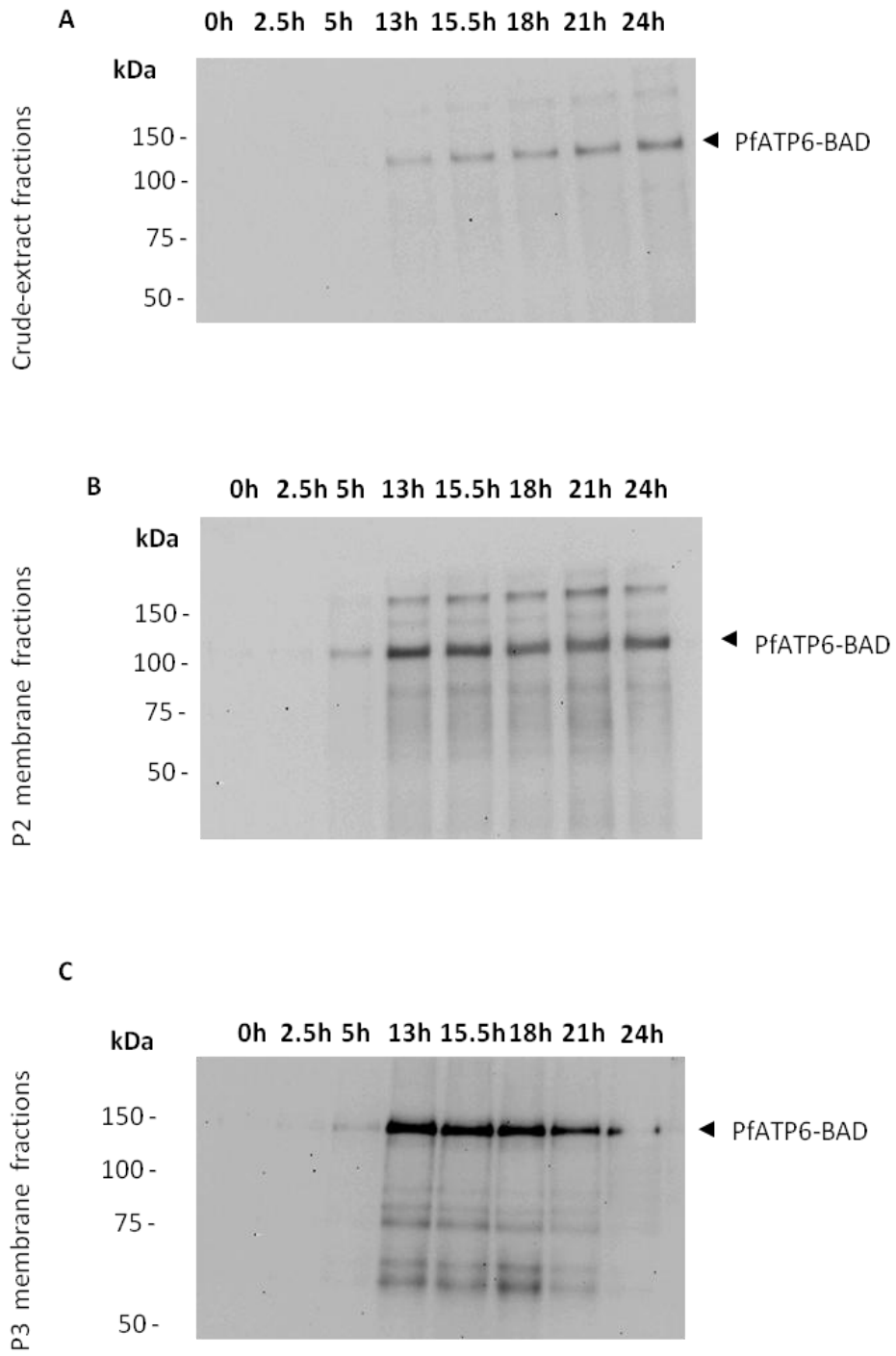


Figure 43 - Kinetic of the expression of PfATP6 in yeast. Western Blot revealed with an anti – PfATP6 specific antibody.

*Fractions of the membrane preparation at each time point: **A** – Crude extract (CE); **B** – membranes from the intermediate speed centrifugation (P2); **C** – Light membrane fractions from the high speed centrifugation (P3). The time points correspond to hours after the first induction, see Figure 41. Detected protein PfATP6-BAD (148 kDa).1µg of total protein is deposited. It is important to note that the protein quantity between each western blot is not comparable because these are independent experiments and the pictures were taken with different exposition times.*

I.5.2 –PfATP6 purification protocol – thrombin cleavage

The purification of PfATP6 was previously established (Cardi et al., 2010b). The description of this procedure can be found in section *I.5 Improvement of PfATP6 expression and purification protocol* and in Figure 40. In the pYeDp60-PfATP6-BAD construction, a thrombin cleavage site is present, which enables elution from the streptavidin-Sepharose resin.

After expression in yeast and light membrane preparation, the P3 fractions have to be washed. During yeast growth in a fermentor and PfATP6 expression phase, there is production of an amount of undesired soluble biotinylated proteins, such as acetyl CoA carboxylase, Pyruvate carboxylase and Arc1p. The expression of these proteins might be due to the modification of yeast metabolism to produce more membranes, and hence the higher expression of proteins involved in fatty acid metabolism (like pyruvate carboxylase, a mitochondrial protein, and acetyl CoA carboxylase, a protein associated with the endoplasmic reticulum) and these undesired expressions are higher in fermentors. These proteins come associated with the light membrane fractions after membrane preparation. To avoid binding of these undesired biotinylated proteins to the streptavidin-Sepharose resin, occupying possible binding sites of PfATP6, we proceed to a washing of the P3 membranes. These are homogenized with a Potter-like homogenizer in a high KCl solution (0.5 M, proved to be efficient in removing these soluble proteins and necessary for Ca^{2+}-ATPases, preventing deactivation and aggregation) and β-mercaptoethanol (reducing agent that protects proteins from oxidation). This was shown to be efficient in removing these soluble proteins and consequently, increase the quantity of PfATP6 bound to the resin (Cardi, 2009).

This procedure of washing the P3 membranes can be observed in lane Tw and Sw where a lot of the Acetyl CoA Carboxylase and the Pyruvate Carboxylase are eliminated in the supernatant of the wash (Sw) (Figure 44 A). PfATP6 is used at a concentration from 5 to 10 mg/ml for solubilization in excess of detergent, a Potter homogeneizer is used to increase the solubilization. However, the solubilization does not seem complete (Ts contains a lot more PfATP6 than the solubilized fraction present in the supernatant Ss). After solubilization, PfATP6 present in the supernatant (Ss) is completely bound to the streptavidin – Sepharose resin (R0), as nothing seems to be lost in the non-retained fraction (NR). After 30 minutes of thrombin cleavage (R30) we can see that almost all PfATP6 is devoided of the BAD domain. A second addition of thrombin left for 30 minutes more (R60) does not seem to change much the cleavage (Figure 44 B and Figure 45 A). We can only see non cleaved PfATP6-BAD on resin after elution (R* Figure 44 B). The elution steps (E1, E2 and E3) allow the collection of purified PfATP6, though this procedure is incomplete as there still remains PfATP6 on the resin (R*). Eluted fraction 1 (E1) possesses the highest amount of PfATP6, although E2 and E3 still collect a lot of purified protein. Eluted fractions are pooled (Figure 45 B) and are concentrated on a Centricon® filter ([c], Figure 44 B and Figure 45 B). After this concentration phase we obtain a rather pure protein concentrated at a factor 18 ([c], Figure 44 B and Figure 45 B). Unfortunately, we lose some of PfATP6 in the flow-through of the Centricon® filter (FT) (Figure 44 B). The concentrated PfATP6 is kept in 40% glycerol, that protects membrane proteins from degradation, and are instantaneously frozen in liquid nitrogen and kept at -80ºC. At last we can quantify the purified PfATP6 by SDS-PAGE and Coomassie blue staining. Purified and concentrated PfATP6 is diluted and deposited to be compared with a range of known concentrations of rabbit sarcoplasmic reticulum SERCA1a (SR) (Figure 45 B).

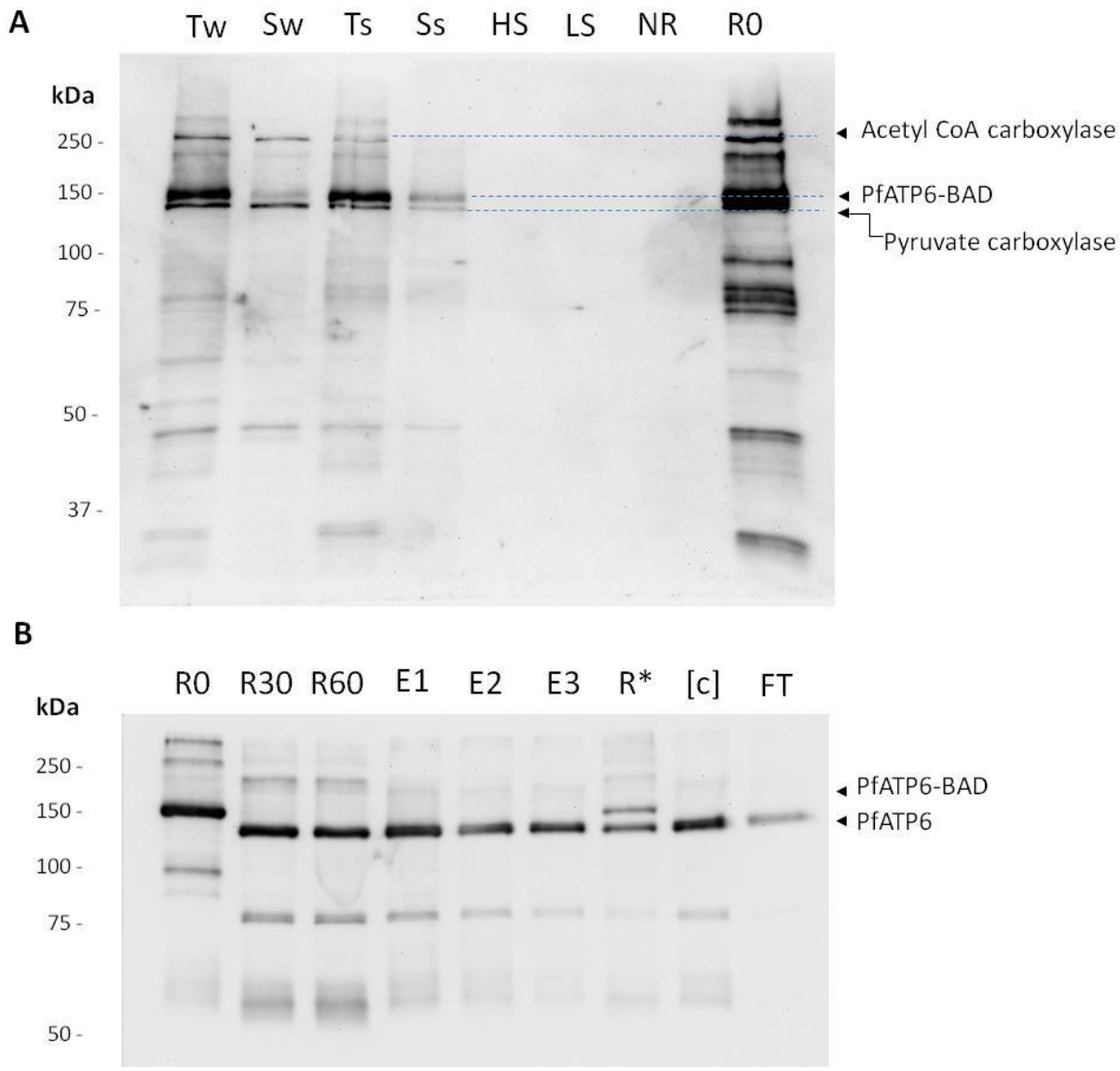


Figure 44 - Purification of PfATP6 followed by Western Blot.

A – *Western blot revealed with avidin peroxidase probe (Biotinylated proteins);* ***B –*** *Western blot revealed with PfATP6 specific antibody. 0.7 µl of each sample was loaded. Molecular mass markers were deposited and are indicated in the figure.* ***A);*** *Tw – total wash of P3 membranes; Sw –discarded supernatant after P3 membrane wash; Ts – total solubilization; Ss – Supernatant from solubilization; HS – High-salt wash; LS – Low-salt Wash; NR – non-retained fraction on resin; R0 - streptavidin-Sepharose resin with solubilized light membrane before cleavage.* ***B)*** *R0 - streptavidin-Sepharose resin with solubilized light membrane before cleavage; R30 – resin after 30min of cleavage; R60 – resin after 60min of cleavage; E1, E2 E3 – first, second and third elution fractions; R* - streptavidin-Sepharose resin after elution; [c] - eluates after 18 fold concentration; FT - flow-through during concentration. Detected proteins: Acetyl CoA Carboxylase (250 kDa), Pyruvate carboxylase (120 kDa), Arc1p (45kDa), PfATP6-BAD (148 kDa), PfATP6 (139 kDa).*

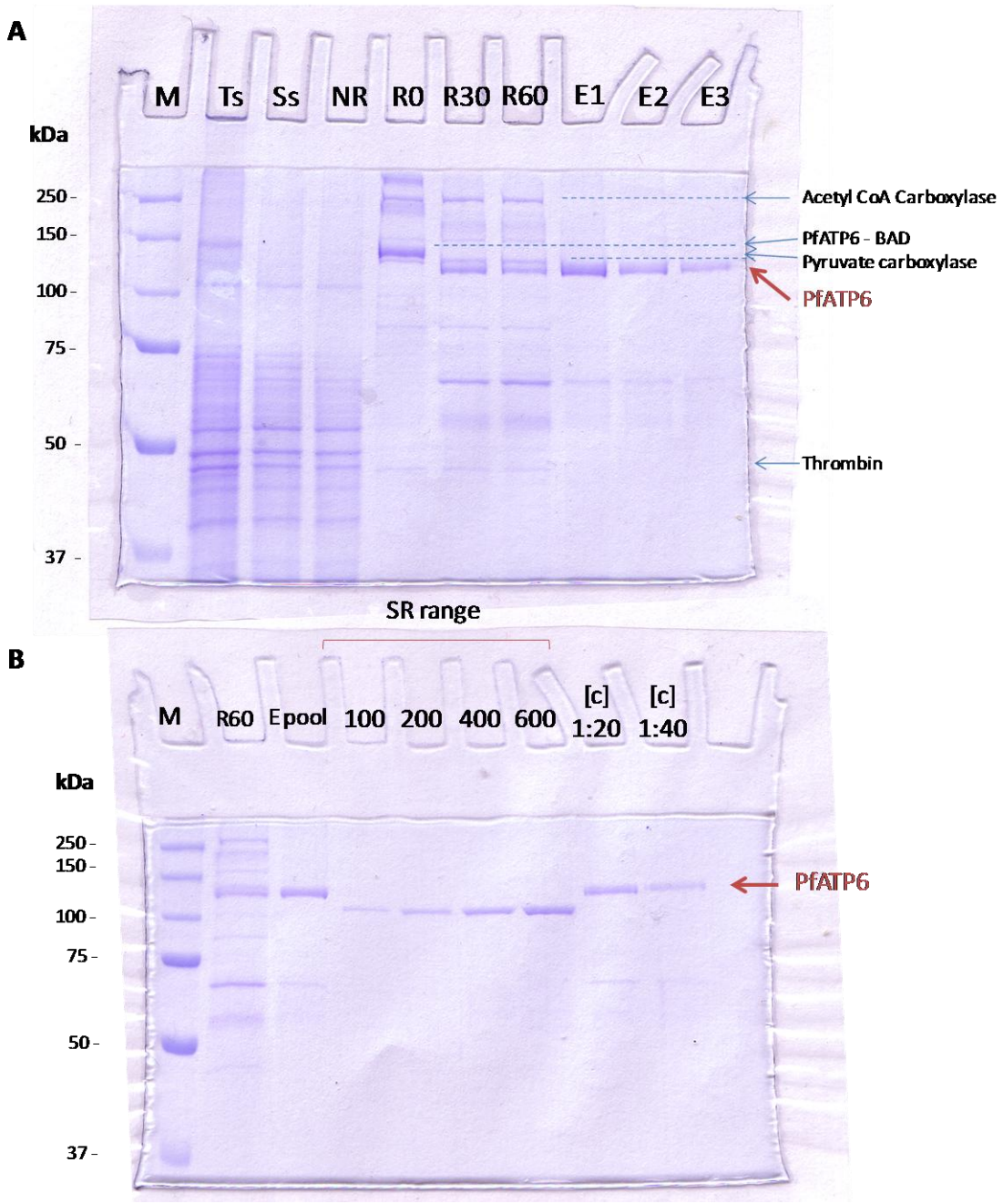


Figure 45 - Purification following and quantification of PfATP6 by SDS-PAGE and Coomassie blue staining.

A) *M – Molecular mass markers; Ts – total solubilization; Ss – Supernatant from solubilization; NR – non-retained fraction on resin; R0 - streptavidin-Sepharose resin with solubilized light membrane before cleavage; R30 – resin after 30min of cleavage; R60 – resin after 60min of cleavage; E1, E2, E3 – first, second and third elution fractions.* ***B)*** *R60 – resin after 60min of cleavage; E pool – first, second and third elution fractions pooled together; Sarcoplasmic reticulum from rabbit muscle containing SERCA1a (SR) was loaded at final concentrations of 100ng, 200 ng, 400 ng and 600 ng; - [c] 1 : 20 – concentrated protein diluted 20 times; [c] 1 : 40 - concentrated protein diluted 40 times. We obtained PfATP6 concentrated at a factor 18. Detected proteins: Acetyl CoA Carboxylase (250 kDa), Pyruvate carboxylase (120 kDa), Arc1p (45kDa), PfATP6-BAD (148 kDa), PfATP6 (139 kDa).*

We obtained here 375 µg of purified and concentrated PfATP6 per 1 L of yeast culture. This amount is higher than obtained in the past (160 µg of PfATP6 per L of culture was obtained by Cardi et al., 2010a). The final yield of PfATP6 purification with the protocol we established was of ~30% (compared to the amount of biotinylated and solubilized PfATP6 expressed in P3 membranes), which is comparable to the values obtained with the previous protocol (D. Cardi PhD thesis (Cardi, 2009), see Table 11). This can be explained by the previous observation that ~30 % of PfATP6 is biotinylated, 25% of biotinylated PfATP6 is solubilized; and that ~20% of PfATP6 stays bound to the resin after elution (observations described in D. Cardi's PhD thesis (Cardi et al., 2010a). The protein purified with the previous protocol (Cardi et al., 2010a) was described to be pure at 70%.

	Yeast harvested	Total protein in P3 membranes	Amount of PfATP6 in P3 membranes	Amount of solubilized PfATP6	Amount of purified PfATP6	Purification yield
Old protocol	50 g/L	400 mg	2.4 mg	600 µg	160 µg	26%
New protocol	50 g/L	300 mg	4.5 mg	1120 µg	375 µg	33%

Table 11 – Comparision of the purification yield between the newly established protocol and the precedent one.

The amount of total protein was calculated with a BCA assay (see Material and Methods section), and the amount of PfATP6 present in P3 membrane fractions was calculated by western blot with an avidin peroxidase probe.

Some steps of the purification protocol can be improved, such as: the solubilization of PfATP6, the cleavage steps, the elution phase, and the concentration phase.

- For the solubilization, perhaps different amount of detergent or another detergent would be worth to try.
- The cleavage could be stopped at 30 minutes and one addition of thrombin, as a second addition does not seem useful. The remaining non-cleaved PfATP6-BAD fraction, seen on the resin after elution (R*, Figure 44 B), is probably not such a big amount, as what we observe is concentrated on the resin and not diluted in the elution fractions, and resin samples are difficult to load onto the gel in a precise amount. However, if this step can be improved it is probably not in the cleavage time nor by increasing the amount of thrombin used. This incomplete cleavage can also be due to bad accessibility of the thrombin cleavage site. Maybe using a different protease would help, like for instance a TEV cleavage site (see section *I.5.3 –Improvement of PfATP6 purification protocol – TEV cleavage*).
- The elution does not appear complete either, again we observe PfATP6 still on the resin after elution (R*, Figure 44 B). It would maybe be useful to try to elute more fractions or try different buffers with higher salt concentrations. However, it is also possible that the remaining PfATP6 is not cleaved or is misfolded, and hence will not be usefull to recuperate.
- The concentration phase is difficult to improve as we always lose protein in the Centricon® filters (also observed with other protein purified in the laboratory). As we always lose the same absolute quantity of purified protein, it is preferable to set up for the purification of larger than smaller amounts of PfATP6, this way we should increase the final yield of the purification.

Even though some points could be slightly improved, this procedure already enables us to purify PfATP6 and obtain a sufficient quantity of protein, higher than previously obtained (Cardi et al., 2010b), enabling our subsequent experiments. As such, we decided to focus our energies in improving another aspect of the purification: the reduction of the procedure cost. For this we aimed to first change the thrombin cleavage site to a TEV cleavage site. As we can produce TEV in *E. coli*, this would significantly reduce the cost linked to cleavage.

I.5.3 –Improvement of PfATP6 purification protocol – TEV cleavage

In order to try to lower the cost of purification, we intended to substitute the thrombin cleavage site between *PfATP6* and *BAD,* which allows the elution of PfATP6 during purification (see above). For this a DNA construction was performed that successfully replaced the thrombin cleavage site to a TEV cleavage site (Figure 46). TEV (Tobacco Etch Virus) protease has a very stringent sequence specifity recognition and has been frequently used to remove affinity tags from recombinant proteins, for purification purposes.

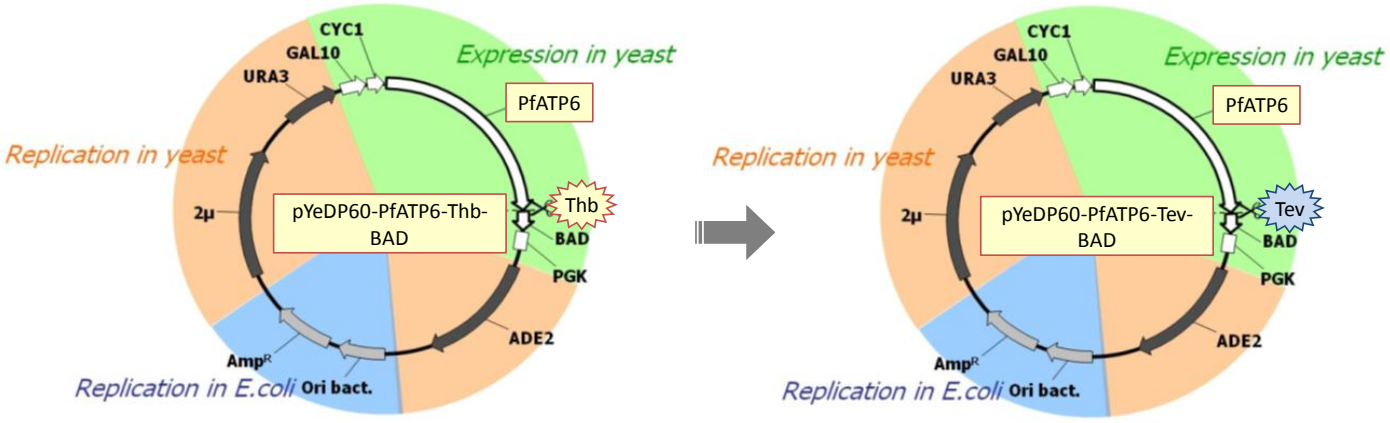


Figure 46 - Plasmid construction for pYeDp60 - PfATP6 – BAD, with different protease cleavage sites (thrombin and TEV).

Cloning and amplification in Escherichia coli (light grey): Ori bact - bacterial replication origin; Amp ^{R} - gene coding for β -lactamase to allow resistance to ampicillin (selection marker). Amplification in yeast (dark grey): ADE2 - auxotrophy selection marker for adenine; URA3 - auxotrophy selection marker for uracil; 2µ - yeast replication origin. Expression (white): GAL10-CYC1 - fusion promoter of the inducible part of GAL10 and RNA polymerase binding part of CYC1; PGK - phosphoglycerate kinase terminator sequence; PfATP6 coding sequence; Thb – thrombin protease, TEV (Tobacco Etch Virus) protease - sequence coding for a protease cleavage site; BAD - biotin acceptor domain. PfATP6, protease cleavage, and BAD sequences were cloned in the same coding frame to allow expression of the fusion PfATP6-BAD.

After sequence verification, we transformed yeast W303.1b/Gal4_2 with the different constructions. We then proceeded to their expression and we performed the membrane preparations in order to

purify the PfATP6 issued from both constructions. We aimed to verify if the modification of the thrombin cleavage site to a TEV site would modify in any way the expression and purification of PfATP6. The results are shown in Figure 47 and Figure 48, obtained by western blot with an avidin peroxydase probe and a specific PfATP6 antibody, respectively. The major difference in the expression of PfAT6 between the two constructions is that pYeDp60-PfATP6-thb-BAD (thrombin cleavage site construction) expresses PfATP6 at a higher level in P2 membranes (compare lane P2 Figure *47* A and B). We can see in Figure *47* A, that P3 membranes express PfATP6 in the same quantity previously obtained with the same construction (lane P3* of Figure *47* A). pYeDp60-PfATP6-TEV-BAD (TEV cleavage site construction) seems to express PfATP6 slightly less than pYeDp60-PfATP6-thb-BAD (compare lane P3 and P3* Figure *47* B).

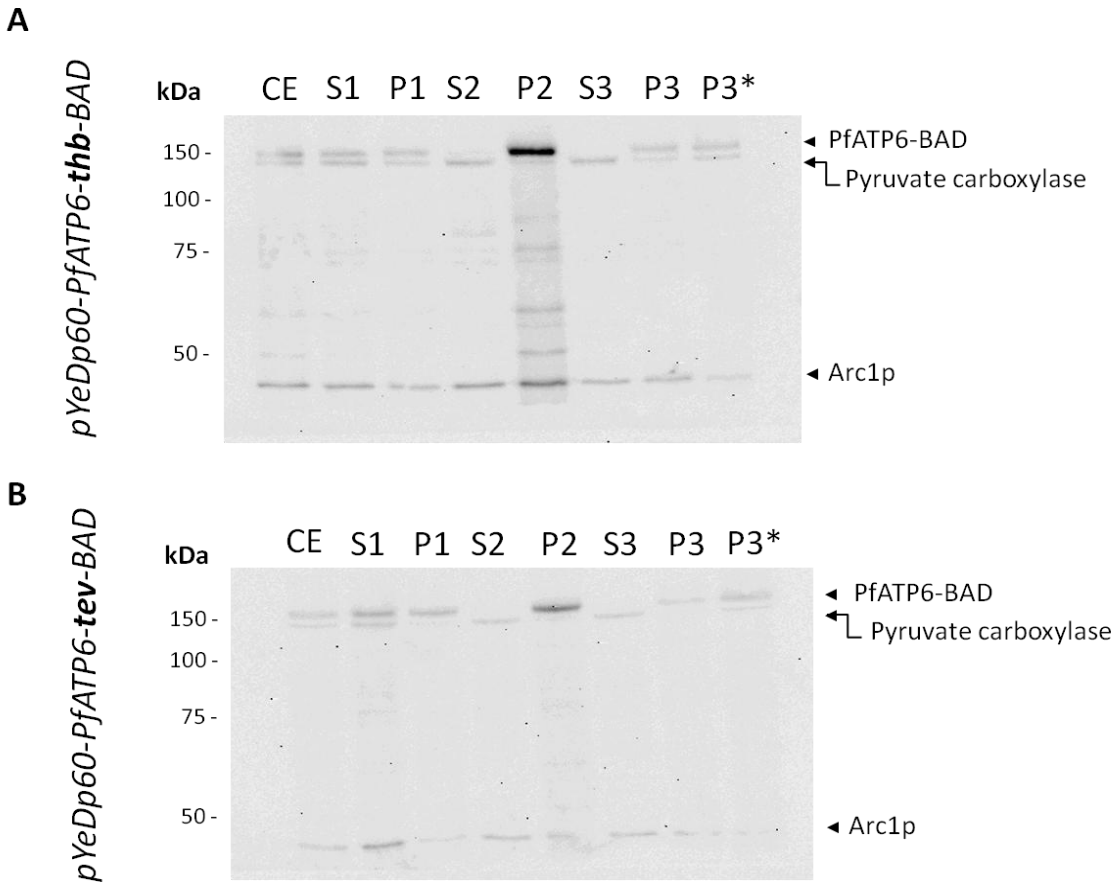


Figure 47 - Expression of PfATP6 in yeast. Western blot with revealed with avidine peroxidase probe (biotinylated proteins).

*Comparison of the expression of PfATP6-**thb**-BAD **(A)** and PfATP6-**TEV**-BAD **(B)**. Following of membrane preparation **:** CE – crude extract; S1 – supernatant from the low speed centrifugation; P1 – pellet from the low speed centrifugation; S2 – supernatant from the intermediate speed centrifugation; P2 – pellet from the intermediate speed centrifugation; S3 – supernatant from the high speed centrifugation; P3 – pellet from the high speed centrifugation; P3* - P3 from a previous membrane preparation. 1µg of total protein is deposited. It is important to note that the protein quantity between each western blot is not comparable because these are independent experiments and the pictures were taken with different exposition times.*

The purification of both constructions undergoes as expected (Figure 48). Cleavage with either thrombin or TEV is complete, even if the cleavage times are not comparable as with thrombin cleavage is undertaken for 60 minutes and overnight for TEV (compare E1, E2 and E3 samples on Figure 48 A and B). Elution is done normally, with a higher amount of PfATP6 in E1 than in E2 and E3 (E1, E2 and E3 samples on Figure 48 A and B). However, when we cleave with TEV, we need to eliminate this protease from purified PfATP6, because we cannot inactivate it as we do with thrombin by PMSF addition. For this, we perform an affinity chromatography on a nickel resin column (Ni-NTA) that will specifically retain TEV due to the presence of a His-tag on the protease. At this stage we realized that all the PfATP6 cleaved with TEV remains on the Ni-NTA resin, and we didn't recuperate purified protein in the elution fractions (Epool and R*-NiNTA amples on Figure 48 B). It is important to note that resin samples are difficult to load, hence the comparision of the quantites with other samples is not accurate.

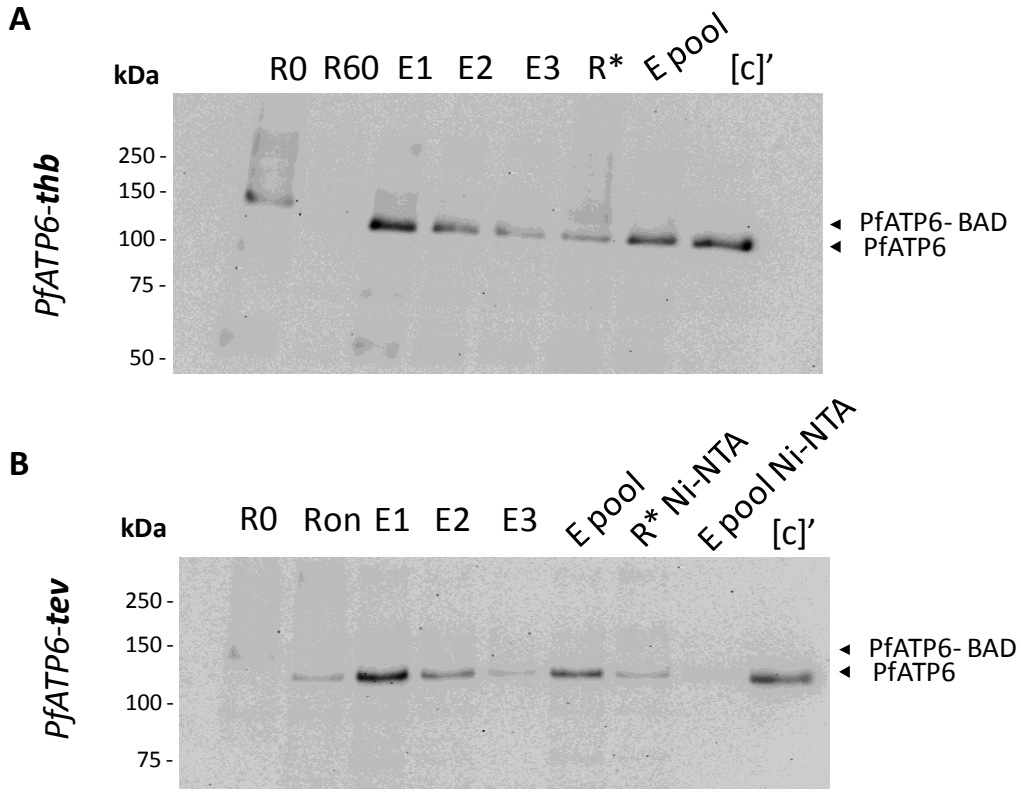


Figure 48 - Purification of PfATP6, comparison of thrombin and TEV cleavage. Western Blot with PfATP6 specific antibody.

A) *PfATP6 purification via thrombin cleavage. R0 - streptavidin-Sepharose resin before cleavage; R60 – resin after 60 minutes thrombin cleavage; E1, E2, E3– first, second and third elution; R* – resin after elution, E pool – elution fraction pooled; [c]' – PfATP6 purified and concentrated from a previous preparation.* ***B)*** *PfATP6 purification via TEV cleavage. R0 - streptavidin-Sepharose resin before cleavage; Ron – resin after overnight TEV cleavage; E1, E2, E3 – first, second and third elution; E pool – elution fraction pooled, R* Ni-NTA – resin Ni-NTA for TEV removal; E pool Ni-NTA – E pool after Ni-NTA; [c]' - PfATP6 purified and concentrated from a previous preparation. It is important to note that the protein quantity between each western blot is not comparable because these are independent experiments and the pictures were taken with different exposition times. 1 µl of each sample was loaded.*

We performed, for both purification processes, an ATPase activity measurement just after elution, before freezing the fractions. With this we aimed to compare the activity of both proteins, to verify if the modification of the cleavage site affected in any way the activity of our protein. We obtained an expected activity for PfATP6 cleaved with thrombin (0,5 µmol ATP hydrolysed.mg^{-1} ATPase.min^{-1}). We observed no activity for PfATP6 cleaved with TEV after Ni-NTA column, but the protein was active before passing on Ni-NTA resin. This result was consistent with a loss of a great amount of PfATP6 that we could not retrieve in the elution.

The BAD domain, and hence the cleavage site, are localized at the C-terminal end of the protein. For thrombin cleavage this never caused any problem. However, TEV protease has a cleavage site composed of 7 amino acid residues and is cleaved asymmetrically (ENLYFQ\S). After cleavage, the N-terminal end is bigger than the C-terminal, and we thus believe that TEV stays attached to this end, that happens to be the end still linked with the protein, in this construction. This has also been observed with other proteins purified in our laboratory (Hassina Azouaoui and Guillaume Lenoir personal communication). In accordance with this observation, it was also noted that upon crystallization, the larger fragment of the peptide substrate originated by the cleavage was co-crystallized with TEV protease, as it was still bound to the enzyme active site (Phan et al., 2002).

One way to solve the problem of the TEV protease maintained attached to the cleaved protein is to change the BAD domain and TEV cleavage site to the N-terminal end of the protein, this way the larger fragment of the cleavage site will stay attached to the resin and hence we can hope that TEV also. An increased salt concentration in the elution buffers may also be attempted to detach TEV from PfATP6. However, based on previous experiments with other proteins in the laboratory, the concentration of TEV used has revealed to be critical and if in the good conditions, TEV may not stay attached to PfATP6 even if the cleavage site is in C-terminal of the protein (Hassina Azouaoui and Guillaume Lenoir personal communication). TEV protease removal attempts were performed using a Ni-NTA resin (agarose matrice). It has been observed in the laboratory with other membrane proteins (Drs2p-Cdc50 complex, Hassina Azouaoui personal communication) that this resin also binds aspecifically to the proteins. The same was not observed when TEV was removed with a Ni-TED resin (silice matrice), hence it will be interesting to try this resin for TEV removal from purified PfATP6. It was also reported that when the purification tag and the cleavage site are localized at N-terminal, the solubilization of another membrane protein is better (Drs2p-Cdc50, Guillaume Lenoir and Hassina Azouaoui, personal communication). With all these observations, we have chosen to modify the terminal end of BAD and the TEV cleavage site to the N-terminal end. However this work is still in progress.

Chapter II

Expression of PfAdT in view of new inhibitors research

II. 1 – Preamble

PfAdT, the adenylate translocase of *P. falciparum,* has previously been object of the PhD project of Valerie Razakantoanina in 2008 and of two publications (Rakotomanga et al., 2004; Razakantoanina et al., 2008).

As described before, one of the objectives of this project was to produce this protein in yeast for structural studies and further proteoliposome reconstruction for functional studies. For this, we inserted the *pfadt* gene (given by Pr. I. Florent) into the yeast expression vector pYeDp60, with a His-tag and a BAD domain at the N-terminal end and a TEV cleavage site, for purification. This will enable expression in yeast and further purification of this protein.

We also intended to find new inhibitors to be tested for their antiplasmodial activity. To achieve this, a functional test to measure PfAdT activity has to be developed. To overcome the challenges of reconstructing PfAdT in proteoliposomes, we decided to use a previously described methodology for activity measurement of this protein (Razakantoanina et al., 2008). Expressing PfAdT on the plasma membrane of *E. coli* strain C43 (DE3) allows a direct monitoring of the uptake of radiolabeled ATP by the cells (Heimpel et al., 2001). With the optimization of this procedure we expect to test molecules for their ability to inhibit PfAdT. For this we inserted *PfAdT* gene into a commercial *E. coli* expression vector (pET20b - Novagen), that has been previously modified and adapted for the expression of the human ATP/ADP carrier (hAAC). This vector possesses a T7 – IPTG inducible promoter, and a Maltose Binding Protein at N-terminal of PfAdT.

For functional studies we aimed to express a *wild type* gene of *PfAdT* and a mutant *PfAdT_K24I*. This mutated position is the corresponding position previously described in bAAC (K22) to be essential for the ATP/ADP carrier activity (Nury et al., 2006; Ravaud et al., 2012).

In this section we will discuss the results relative to the expression of PfAdT in yeast, the expression of PfAdT in *E. coli*, and finally the concluding comments and perspectives for this work.

II.2 – Expression of PfAdT in Yeast

II.2.1 - Construction of pYeDp60 – His6 – BAD – TEV– PfAdT

The mutant *PfAdT_K24I* was originated from the wild type gene by direct mutagenesis. The gene sequences were inserted into pYeDp60 with a His_6-Tag and the BAD domain at the N-terminal end of the final protein, followed by a TEV cleavage site (Figure 49). The BAD domain is localized at N-terminal of the protein because a good expression of hAAC was obtained by Paul Machillot, in our laboratory. The His_6-Tag and the BAD domain enable detection of the protein by western blot with a His-probe and an avidin-peroxidase probe, respectively.

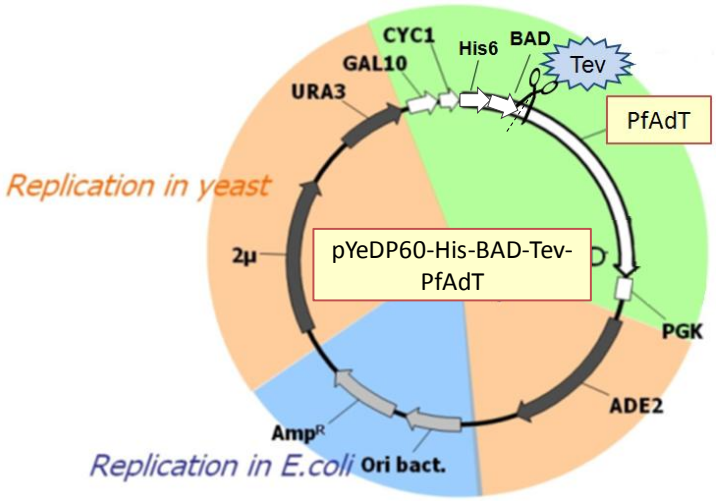


Figure 49 - PfAdT-pYeDp60 vector construction.

Cloning and amplification in Escherichia coli (light grey): Ori bact - bacterial replication origin; Amp ^{R} - gene coding for β -lactamase to allow resistance to ampicillin (selection marker). Amplification in yeast (dark grey): ADE2 - auxotrophy selection marker for adenine; URA3 - auxotrophy selection marker for uracil; 2µ - yeast replication origin. Expression (white): GAL10-CYC1 - fusion promoter of the inducible part of GAL10 and RNA polymerase binding part of CYC1; PGK - phosphoglycerate kinase terminator sequence; PfAdT coding sequence; Protease cleavage site - sequence coding for a protease cleavage site; BAD - biotin acceptor domain. PfAdT, protease cleavage, and BAD sequences were cloned in the same coding frame to allow expression of the fusion PfAdT-BAD.

II.2.2 – Expression of PfAdT_wt and PfAdT_K24I in yeast

After verification of the vector construction by gene sequencing, *Saccharomyces cerevisae* strain W303.1b/Gal4_2 was transformed with the above plasmids. The validation of the transformation was done by expression in minimum medium, as previously described for PfATP6. The positive clones were then cultured in rich medium and PfAdT_wt and PfADT_K24I were successfully expressed in yeast (Figure 50 and Figure 51)

Both PfAdT_wt (Figure 50) and PfAdT_K24I (Figure 51) express in yeast in a satisfactory amount (~1%), compared to the known amount of expression of hAAC in yeast P3 membranes. Though, the wild type form of the protein seems to be expressed in higher amounts than the mutant K24I when revealed with an avidin-peroxydase probe (Figure 50 A), but when revealed with a His-probe (Figure 50 B) PfAdT_K24I seems to express in a higher amount than the wild-type. But it is important not to forget that the detection with avidin-peroxidase detects biotinylated proteins, and the His-probe detects His-tags. Perhaps the expressed proteins are not all biotinylated and these would be detected with the His-probe compared to the detected with the avidin-peroxidase probe, in this case PfAdT_K24I is expressed in higher amount than PfAdT_wt, but the wild type form is proportionally better biotinylated than the mutant. It seems that both forms of PfAdT express in a minor quantity when compared to hAAC expression (Figure 50 B). But the amount of biotinylation of PfAdT_wt is comparable to the amount of hAAC biotinylated (Figure 50 A). However one has to be cautious because these membranes are hardly comparable; as hAAC was expressed in a bioreactor and both PfAdT were expressed in Fernbach flasks, which are known to lead to a lower expression yield (verified with SERCA1a and PfATP6).

During the membrane preparation, there is always an important lost of protein in the intermediate spin pellet (P2), which we usually observe with other proteins (see Chapter 1 of Results and Discussion session, for PfATP6 example). But as discussed before, PfATP6 and SERCA1a are not very active in this membrane environment.

With this experimental procedure we are able to express the wild type form of PfAdT and the mutant K24I. In the future, a protocol for the purification of PfAdT can be established inspired in the procedure already defined to purify hAAC (non published data).

Purifying this protein will be the first step for the investment in the crystallization of PfAdT which will bring important structural information that could allow inhibitors design and hence potential antimalarials discovery.

As presented above, we aimed to find specific PfAdT inhibitors and test for their antiplasmodial activity. We then decided to develop an activity measurement test by expressing PfAdT_Wt and PfAdT_K24I in *E. coli* to directly measure the ATP uptake from whole cells, as previously described by V. Razankantoanina and collaborators in 2008 (Razakantoanina et al., 2008).

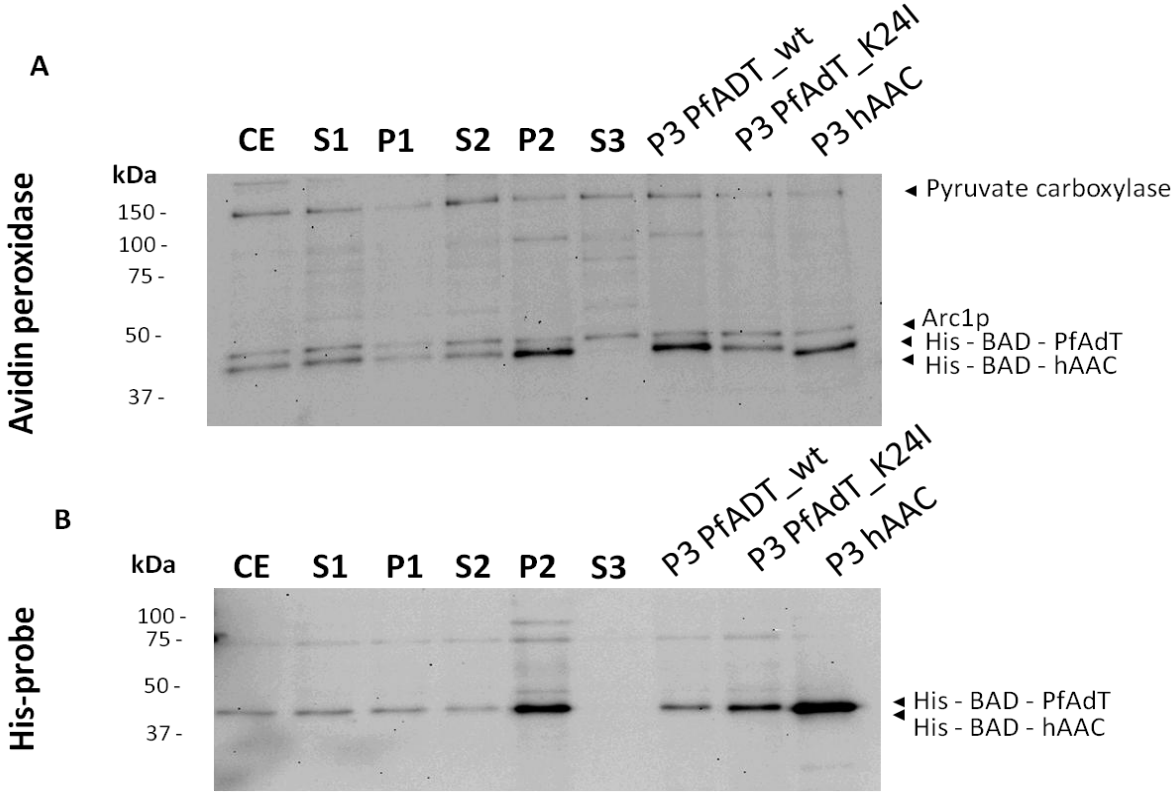


Figure 50 - Expression of PfAdT_wt in yeast.

*Western blot revealed by: **A)** Avidin peroxidase probe; **B)** His-probe. Following of membrane preparation : CE – crude extract; S1 – supernatant from the low speed centrifugation; P1 – pellet from the low speed centrifugation; S2 – supernatant from the intermediate speed centrifugation; P2 – pellet from the intermediate speed centrifugation; S3 – supernatant from the high speed centrifugation; P3PfAdT_wt – pellet from the high speed centrifugation; P3 PfAdT_K24I - P3 from a previous membrane preparation of PfAdT_K24I; P3 hAAC - P3 from a previous membrane preparation of hAAC (obtained by Cédric Montigny and Paul Machillot). 1µg of total protein is deposited.*

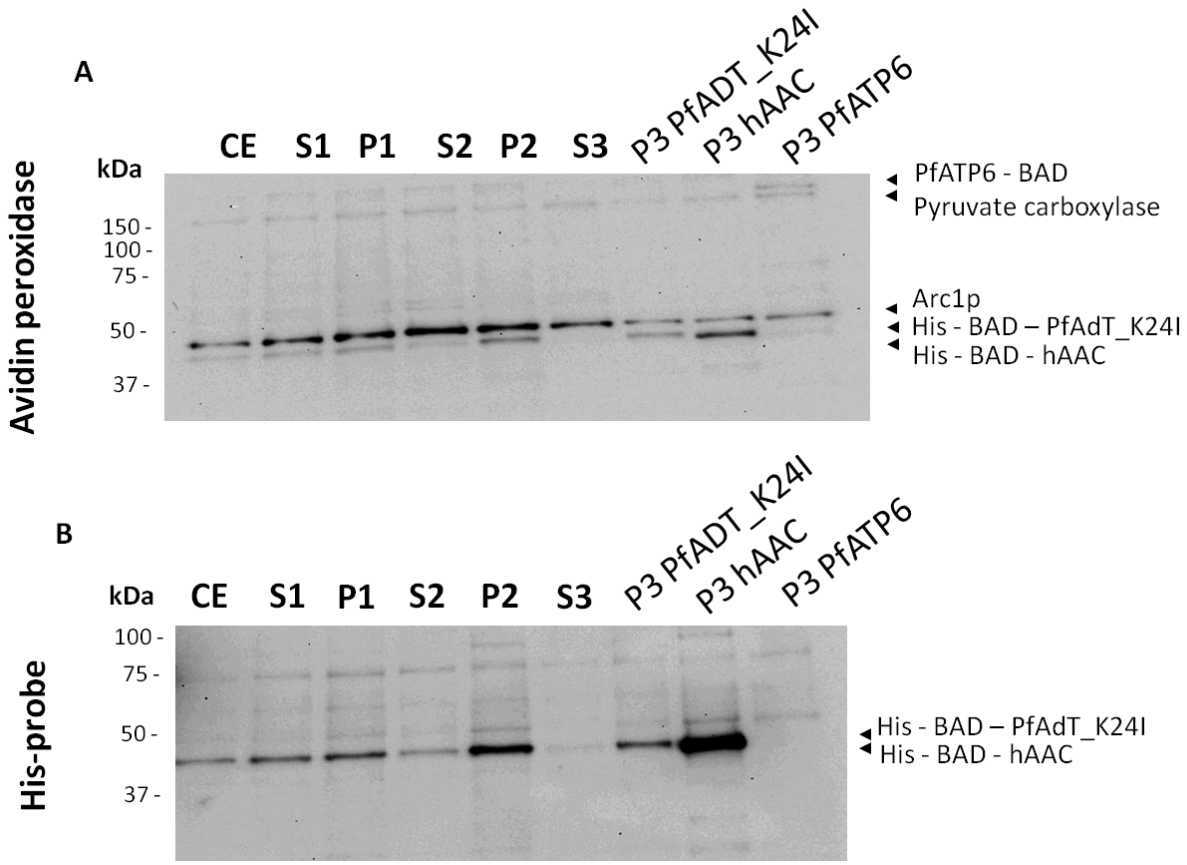


Figure 51 - Expression of the mutant PfAdT_K24I in yeast.

*Western blot revealed by: **A)** Avidin peroxidase probe; B) His-probe. Following of membrane preparation : CE – crude extract; S1 – supernatant from the low speed centrifugation; P1 – pellet from the low speed centrifugation; S2 – supernatant from the intermediate speed centrifugation; P2 – pellet from the intermediate speed centrifugation; S3 – supernatant from the high speed centrifugation; P3 PfAdT_K24I – pellet from the high speed centrifugation; P3 hAAC - P3 from previous membrane preparation of hAAC (obtained by Cédric Montigny and Paul Machillot). P3 PfATP6 - P3 from previous membrane preparation of PfATP6. 1µg of total protein is deposited.*

II.3.1 –- Construction of pET20b – MBP – Thb – PfAdT

PfAdT_K24I and PfAdT_wt were inserted in a pET20b vector. The commercial pET20b vector contains a N-terminal pelB signal sequence for potential periplasmic localization, and a T7-IPTG inducible promoter. At the N-terminal of PfAdT a Maltose Binding Protein (MBP) sequence was introduced, separated by a thrombin cleavage site. This sequence will promote the export of the MBP- fusioned -PfAdT protein to the periplasm and favor the expression of PfAdT on the bacteria outer membrane (Figure 52). MBP can be detected with a specific MBP monoclonal antibody (Biolabs).

Expression of PfAdT in *E. coli* plasma membrane (Razakantoanina et al., 2008) will enable functional studies that could help characterizing this poorly studied *P. falciparum* transporter. The mutant K24I can give insights into ADP/ATP exchange but might also be a good negative control for inhibitors screening.

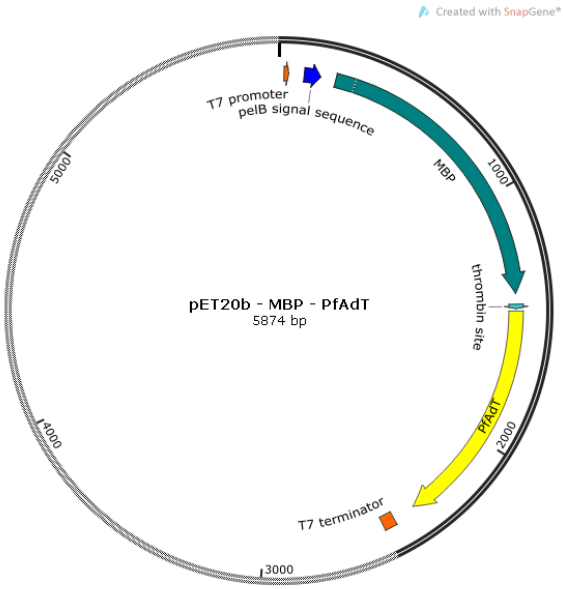


Figure 52 - PfAdT_Wt and PfAdT_K24I in pET20b vector.

Cloning and amplification in Escherichia coli (light grey): Ori bact - bacterial replication origin; Amp ^{R} - gene coding for β -lactamase to allow resistance to ampicillin (selection marker); T7 promoter – IPTG inducible; pelB signal sequence for potential periplasmic localization; MBP – Maltose-Binding-Domain; thrombinn cleavage site - sequence coding for a thrombin cleavage site; PfAdT – gene sequence coding for PfAdT; 6xHis – sequence coding for a 6 histidine tag; T7 terminator – end of the reding frame. pelB signal, MBP, PfAdT, thrombine cleavage, and 6xHis sequences were cloned in the same coding frame to allow the expression of the fusion PfAdT-MBP.

II.3.2 - Expression of PfAdT_wt and PfAdT_K24I in *E. coli*

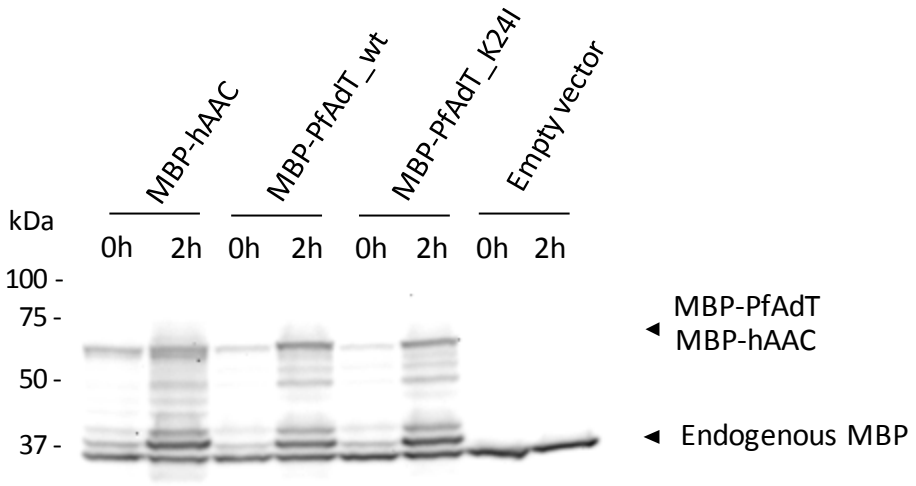


Figure 53 - expression of PfAdT and hAAC in E.coli.

Western blot revealed with a MBP monoclonal antibody that detects MBP-hAAC (78.2 kDa), MBP-PfAdT_wt and MBP-PfAdT_K24I (78.9 kDa) and also E. coli endogenous MBP (45.2 kDa). MBP-Maltose-Binding Protein fusion,. 0h – E. coli before IPTG induction; 2h – E. coli after 2h of IPTG induced protein expression. The DNA construction here used for MBP-hAAC expression was given by S. Ravaud. 0.1 final OD_{600nm} was deposited for each sample.

After several attempts of the protocol optimization, the expression of PfAdT_wt and PfAdT_K24I in *E. coli* strain C43 (DE3) was obtained (Figure 53). On the western blot above (Figure 53), we can observe the MBP fused PfAdT localized at 78.9 kDa (corresponding to a size of 45.2 kDa for MBP and 33.7 kDa for PfAdT). The bands underneath might be due to degradation due to sample preparation and heating up to 100ºC before gel loading. It is important to note that before induction (lanes indicated with 0h), there is already expression of our proteins, indicating expression leakage from this system. However, when we compared to *E. coli* transformed with an empty vector, only the endogenous *E. coli* maltose binding protein can be seen by western blot. This indicates that indeed we are able to express hAAC, PfAdT_wt and PfAdT_K24I using this system.

The next step will be to verify if PfAdT_wt is active, and PfAdT_k24I inactive as predicted. Then a protocol for radiolabeled ATP uptake measrument can be established. Then known AAC inhibitors can be tested as well as research of new PfAdT specific inhibitors.

Chapter III

Expression of SERCA1a, SERCA-1a_E255L and PfATP6 in Xenopus laevis oocytes and effect of thapsigargin, CPA and artemisinin on the Ca^{2+}-dependent ATPase activity

III.1 – Preamble

As a reminder from the introduction, PfATP6 was described by S. Krishna and collaborators in 2003 (Eckstein-Ludwig et al., 2003) as the target of the potent antimalarial artemisinin. Following mRNA injection of PfATP6 and of the homologous protein SERCA1a from rabbit skeletal muscle in *Xenopus laevis* oocytes, they prepared oocyte membranes. Using these membranes, they provided results of coupled-enzyme ATPase activity tests in which PfATP6 appeared to be sensitive to artemisinin, but not SERCA-1a, so they concluded that PfATP6 is the target of the most used antimalarial worldwide. In 2005 Krishna's team, using the same technique, put forward the idea that a single amino acid (L263) was responsible for the sensitivity of PfATP6 to artemisinins (Uhlemann et al., 2005). Hence, they indicated that after the mutation L263E, PfATP6 becomes insensitive to artemisinin. Conversely they indicated that the equivalent position in rabbit SERCA1a (E255), when mutated to a leucine, makes this protein sensitive to artemisinin. In 2010, it was demonstrated that the purified PfATP6 and SERCA1a mutant E255L (expressed in *Saccharomyces cerevisiae*) were both insensitive to artemisinin (Cardi et al., 2010b). Similar results were obtained with microsomes of COS-1cells expressing the mammalian E255L mutant, being unsuccessful in confirming that the mutation of Glu^{255} to Leu determines the sensitivity to artemisinin (Cardi et al., 2010b).

Despite this demonstration, Krishna and coworkers maintained the hypothesis that PfATP6 is the target of artemisinin (Krishna et al., 2014, 2010; Valderramos et al., 2010).

Our attempts to reproduce Krishna's team so controversed results are the object of a publication that is presented below (in the process of being submitted as a Correspondence to Nature Structural and Molecular Biology).

III.2 - Article - Reappraisal of oocytes experiments on Plasmodium falciparum transporter PfATP6 and SERCA-1a E255L

Stéphanie David-Bosne[1], Michael Jakob Clausen[2,3,4], Hanne Poulsen[2,3,4], Jesper Vuust Møller[2,3,4], Marc le Maire[1,5] and Poul Nissen[2,3,4,5]

[1]Laboratoire des Protéines Membranaires, UMR 8221, Commissariat à l'Energie Atomique (CEA), Université Paris-Sud and Centre National de la Recherche Scientifique (CNRS) Gif-sur-Yvette, France
[2]Centre for Membrane Pumps in Cells and Disease – PUMPKIN, Danish National Research Foundation [3]Department of Molecular Biology and Genetics, Aarhus University, Denmark
[4]Department of Biomedicine, Aarhus University, Denmark
[5]To whom correspondence should be addressed; marc.lemaire@cea.fr **;** pn@mb.au.dk

To be submitted as a Correspondence to Nature Structural & Molecular Biology

Reappraisal of the *Plasmodium falciparum* transporter PfATP6 and SERCA1a-255L as the molecular target of artemisinin)

Stéphanie David-Bosne[1], Michael Voldsgaard Clausen[2,3,4], Hanne Poulsen[2,3,4], Jesper Vuust Møller[2,3,4], Poul Nissen[2,3,4,5] and Marc le Maire[1,5].

[1]Laboratoire des Protéines Membranaires, UMR 8221, Commissariat à l'Energie Atomique (CEA), Université Paris-Sud and Centre National de la Recherche Scientifique (CNRS) Gif-sur-Yvette, France [2]Centre for Membrane Pumps in Cells and Disease – PUMPKIN, Danish National Research Foundation [3]Department of Molecular Biology and Genetics, Aarhus University, Denmark [4]Department of Biomedicine, Aarhus University, Denmark

[5]To whom correspondence should be addressed; marc.lemaire@cea.fr ; pn@mb.au.dk

Malaria is a vector-borne infectious disease caused by a parasite of the *Plasmodium* genus and typically transmitted by the *Anopheles* mosquito, which introduces the parasite to the blood stream of a human host. Malaria is a life-threatening disease causing an estimated 627,000 deaths in 2012 according to the World Health Organization[1].

No vaccine is yet available, but several antimalarial treatments exist of which the most widely used is artemisinin administered together with other antimalarials in Artemisinin Combination Therapies. A decrease in artemisinin sensitivity was recently reported in South-East Asia[2], urging the need for reappraisal of the situation. Understanding the molecular mechanism of action of artemisinin is fundamental for improved diagnosis, administration and further drug development.

PfATP6, the Sarcoplasmic-Endoplasmic Ca^{2+}-ATPase (SERCA) from *Plasmodium falciparum*, has been suggested as the target for artemisinin[3]. PfATP6 is homologous to SERCA1a from animal skeletal muscle, not only in amino-acid sequence, but also in functional properties[4]. Following injection of PfATP6 or rabbit SERCA1a mRNA into *Xenopus laevis* oocytes, Eckstein-Ludwig and coworkers prepared oocyte membranes, and they reported that the ATPase activity of the expressed PfATP6 was inhibited by artemisinin, while the drug had no effect on SERCA1a from rabbit muscle[3]. A single residue in PfATP6, L263, was reported to be critical with an L263E substitution making PfATP6 artemisinin insensitive [5]. Additionally, the reverse substitution in rabbit SERCA1a, E255L, was reported to confer artemisinin sensitivity.

In contrast to the results reported for *Xenopus laevis* oocyte expressed PfATP6 and SERCA1a-E255L, we found that artemisinin had no effect on the ATPase activities of the proteins when expressed and purified in active form from *Saccharomyces cerevisae*[6]. Similarly, we were unable to show any effect on SERCA1a E255L expressed in microsomes of COS-1 cells[6]. PfATP6 is, nonetheless, still being claimed to be the target of artemisinin,[7–10] presumably based on the idea that the *Xenopus laevis* oocyte expression system somehow may be critical for obtaining artemisinin sensitive ATPase [8]. As a result substantial human and economic resources have been spent on large scale sequencing of the PfATP6 gene in world regions with artemisinin resistance. A number of studies have found no association between previously described PfATP6 mutations and artemisinin resistance [11–32], and few reported some association [33–35] but none with L263.

Determining whether artemisinin targets PfATP6 is therefore of major importance for the proper management of resources in the prevention and treatment of malaria. Here, we report the effects of artemisinin on the ATPase activities of PfATP6, SERCA1a and SERCA1a-E255L expressed in *Xenopus laevis* oocytes. We demonstrate Ca^{2+}-dependent and thapsigargin-sensitive ATPase activities of SERCA1a and of SERCA1a-E255L, but both ATPases were insensitive to artemisinin. Furthermore, PfATP6 expression in oocytes was reproduced using the reported protocols, but the PfATP6 expressed in oocytes was inactive and absent from the endoplasmic reticulum membrane fractions (microsomes). We therefore consider it beyond reasonable doubt that artemisinin does not target PfATP6, and we urge that novel strategies be sought to determine artemisinin's molecular mechanism.

Material and Methods The coding sequences of SERCA1a, SERCA1a – E255L and PfATP6 were introduced into the pXOON vector [36] for expression in *Xenopus laevis* oocytes. *In vitro* transcribed mRNA (5 – 30ng) was injected into oocytes, which were maintained 2 – 5 days at 19°C for expression of the proteins[3,37,38].

Membrane preparation was similar to previously described protocols [3,5,38,39]. Oocyte homogenates from 100-150 oocytes were centrifuged at 1000 x *g* for 5 min, and the resulting yolk granule/melanosome pellet was recentrifuged. Supernatants were pooled and centrifuged (100,000 x *g*, 90 min) to obtain a membrane fraction, P2, which was stored at -70°C. Western blotting confirmed the presence of SERCA1a proteins in the oocyte membrane preparations. Highest expression levels were seen at 5 days for both SERCA1a (4% of total proteins) and SERCA1a-E255L (2% of total proteins), while for PfATP6 (4% of total proteins) the same expression level was seen at 2 and 5 days.

We performed ATPase activity tests on oocyte membranes [3,5]. No Ca^{2+}-dependent ATPase activity (measured at room temperature) [6] was seen with 10 μg/ml total protein- which is the amount used in [3]. We hence decided to increase up to 10-fold of total protein concentration (100 μg/ml, corresponding to 4 μg/ml of SERCA1a and 2 μg/ml of SERCA1a-E255L) and even 200 μg/ml, corresponding to 8 μg/ml for PfATP6.

RESULTS

SERCA1a and SERCA1a-E255L expressed in *Xenopus laevis* oocytes are artemisinin insensitive

It was previously reported that artemisinin has no effect on SERCA1a, but inhibits the mutant SERCA1a-E255L with a Ki of 314 ± 109 nM[5], a value later raised to 539 nM[9]. To test if we could reproduce these results, we similarly used *Xenopus laevis* oocytes to express the ATPases (supplementary figure 1), and we could show Ca^{2+}-dependent and thapsigargin (or EGTA) sensitive specific activity in the purified P2 membrane fractions (Figure 1). 50 μM artemisinin did not inhibit wild type SERCA1a, but neither did it show any effect on SERCA1a-E255L (Figure 1 D and F). Using a different protocol to prepare membrane fractions as microsomes for SERCA1a and SERCA1a-E255L, the amount of protein was lower in P3 than in P2 (supplementary figure 4B), but the Ca^{2+}-dependent ATPase specific activity was higher (supplementary figure 2) as also shown previously for yeast expressed SERCA1a [40,41].

The artemisinin used was concomitantly tested directly on live parasites and found to be active at nanomolar concentrations as expected (Isabelle Florent, MNHN Paris, personal communication).

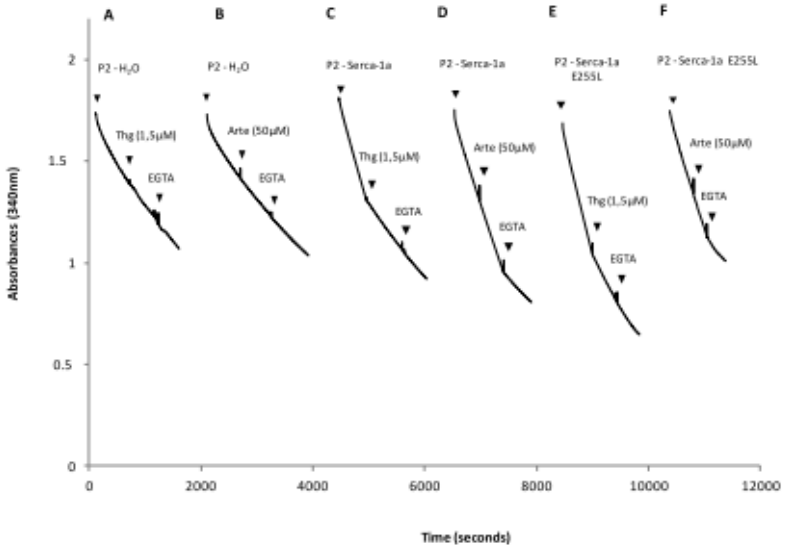


Figure 1 - SERCA1a and SERCA1a–E255L from P2 are sensitive to thapsigargin and EGTA, but not to artemisinin. Effect of 1.5 µM thapsigargin (Thg) or 50 µM artemisinin (Arte) was tested on P2 membrane fractions with 100 µg/ml of total protein prepared from: A and B – oocytes injected with water; C and D – oocytes injected with SERCA1a mRNA; E and F – oocytes injected with SERCA1a-E255L mRNA. The ATPase activity of SERCA1a (curve C) was approximately 2.3 µmol.mg^{-1}.min^{-1} and 1 after Thg addition. For SERCA1a-255L, in curve E, it was 5.3 µmol.mg^{-1}.min^{-1} and 2.7 after Thg addition, and, in curve F, it was 4.3 µmol.mg^{-1}.min^{-1} and 4.1 after artemisinin addition. The background Ca^{2+} sensitive activity of non-expressing oocyte membranes was not significant.

PfATP6 expressed in *Xenopus laevis* oocytes is inactive

In parallel, we expressed PfATP6 in oocytes and prepared membrane fractions as previously reported [3,5]. PfATP6 expression was observed (supplementary figure S3), but we were unable to demonstrate any Ca^{2+}-dependent specific activity (Figure 2). PfATP6 expressed in yeast possesses a specific ATPase activity, which is somewhat lower than that of SERCA1a (1.7 vs. 5.7 µmol·mg-^{1}·min^{-1} respectively[6,42]) so a minimum of 5 µg/ml of this protein would be needed to detect Ca^{2+}-dependent ATPase activity for PfATP6. However, despite using even higher protein concentration (200 µg/ml total protein, corresponding to about 8 µg/ml of PfATP6) than described[3,5], no Ca^{2+}-dependent/EGTA sensitive specific activity was observed, and consequently no inhibition could be demonstrated with cyclopiazonic acid (CPA), a potent inhibitor of PfATP6[6], nor with artemisinin.

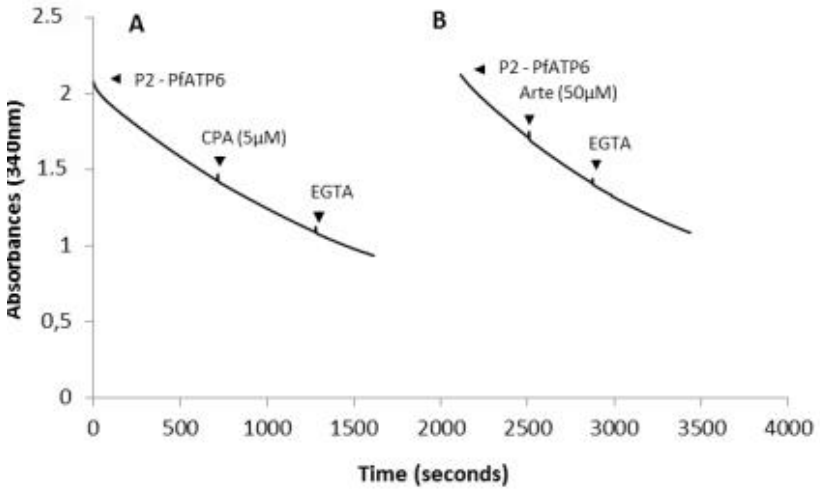


Figure 2 - PfATP6 from P2 is insensitive to CPA, artemisinin and EGTA. Effect of 5 µM of CPA, a potent inhibitor of PfATP6[4], or 50 µM of artemisinin (Arte) was tested on P2 membrane fractions with 200 µg/ml of total protein prepared from oocytes injected with PfATP6 mRNA. 4% of total protein was PfATP6.

Based on previous experience[41], we also tested the effect of adding an inhibitor cocktail for non-P-type ATPases to diminish the background while maintaining the activity of Ca^{2+}-ATPases. Despite several trials, no PfATP6 Ca^{2+}-dependent specific activity could be detected (data not shown). There was no difference between membranes prepared after 2 or 5 days of expression (supplementary figure S3). We also prepared the P3 membrane fraction corresponding to microsomes of endoplasmic reticulum membranes [39]. PfATP6 was absent from the microsomal fraction (supplementary figure 4A). We have noted previously that the fractions corresponding to P2 membranes display a lower Ca^{2+}-dependent ATPase specific activity than P3 endoplasmic reticulum membranes. This suggests that PfATP6 expressed in the oocytes is not maintained in a proper membrane environment (endoplasmic reticulum), most likely explaining why it is inactive. This problem is commonly observed for heterologous expression of membrane proteins; active PfATP6 however is obtained from a yeast expression system [4,6].

Discussion

The results presented here address the controversy on artemisinin's method of action. Despite the use of several different membrane preparations and activity measurement protocols, no specific, Ca^{2+}-dependent ATPase activity of PfATP6 expressed in *Xenopus laevis* oocyte membranes could be demonstrated. Using active and inhibitable PfATP6 expressed in yeast endoplasmic reticulum membranes, we have previously concluded that artemisinin has no effect on PfATP6 [4,6]. The rabbit SERCA1a-E255L mutant was expressed successfully as an active Ca^{2+}-ATPase in oocyte membranes, but showed no artemisinin-sensitivity in our experiments, which contrast claims that this mutant should be artemisinin sensitive [5,9]. The data reported from the Krishna group are challenged by low

signals, large standard deviations, and missing controls [3,5,9]. Therefore, it is possible that the disagreement is more in the interpretation than in the data themselves.

The oocyte expression system has previously proven problematic for characterization of the *Plasmodium* ATPase PfATP4, which too bears sequence homology to SERCA1a. PfATP4 was reported by Krishna et al. to be a Ca^{2+}-dependent ATPase [38], but subsequent experiments in other laboratories have shown that PfATP4 is a Na^{2+}-dependent ATPase [43] and that the oocyte experiments could not be reproduced [44]. We recommend that no presumptions be made on substrate specificities of protist P-type ATPases based on sequence analysis alone, and that the oocyte expression system should only be used with great caution for *Plasmodium* ATPase research.

Our conclusions unfortunately leave open the important question of what artemisinin's mechanism of action although data in the literature point out to interesting new tracks among reported pleiotropic effects (see e.g. [28,45,46]), the answer to which would open new possible strategies to overcome the currently increasing artemisinin resistance. However, despite our negative results on PfATP6 as a target for artemisinin, *Plasmodium* ATPases remain attractive drug targets: even if the complete genetic validation has not been performed a PfATP6 knockout in *P. falciparum* is reportedly lethal [47], and a class of the most promising new antimalarials, the spiroindolones, are potent PfATP4 inhibitors [44].

Supplementary data

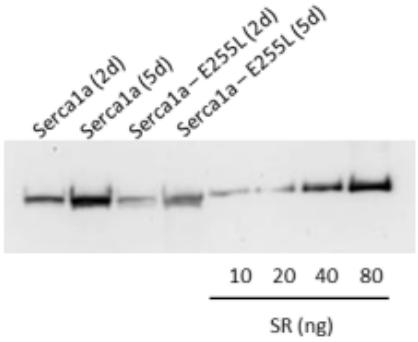


Figure S1 - Expression profiles of SERCA1a and SERCA1a–E255L after western blotting. *Xenopus laevis* oocytes were injected with SERCA1a mRNA or SERCA1a–E255L mRNA. 1 µg of total final protein from P2 membrane fractions was loaded. 2d – 2 days; 5d – 5 days; SR - SERCA1a ATPase preparation from rabbit skeletal muscle. See also fig. S4

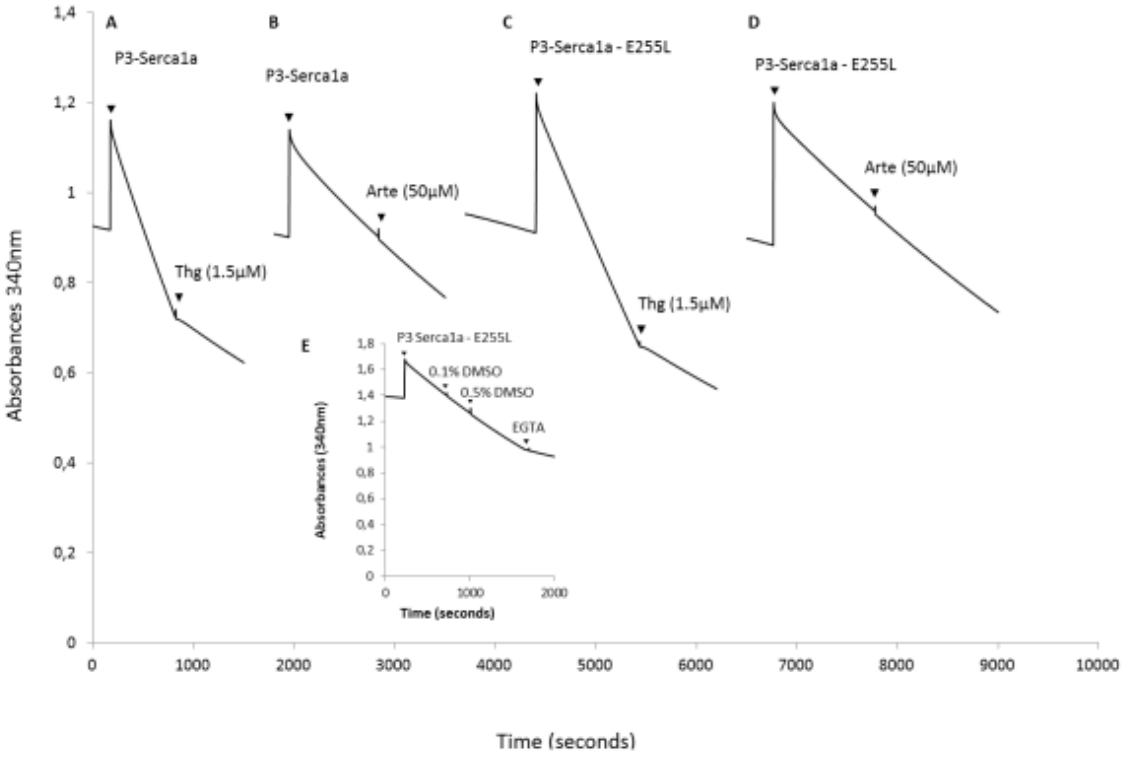


Figure S2 - SERCA1a and SERCA1a–E255L from P3 are sensitive to thapsigargin, but not to artemisinin. Effect of 1.5 µM of thapsigargin (Thg) or 50 µM of artemisinin (Arte) or various concentrations of DMSO as controls was tested on P3 membrane fractions with 100 µg/ml of total protein prepared from: A and B – oocytes injected with SERCA1a mRNA; C, D and E – oocytes injected with SERCA1a-E255L mRNA. The ATPase activity of the SERCA1a and SERCA1a-E255L was ~5 µmol.mg^{-1}.min^{-1}, determined by the difference between the slope before and after the reaction was stopped with EGTA addition.

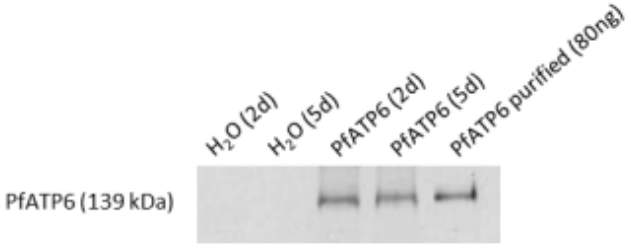


Figure S3 - Expression profiles of PfATP6 in oocyte membranes. *Xenopus laevis* oocytes were injected with water as control and mRNA for PfATP6. Purified active PfATP6 based on yeast expression was prepared according to reported protocols[6,48]. The oocyte membranes were prepared as previously described[3,5] and deposited at 1 µg of total final protein. 2d – 2 days; 5d – 5 days.

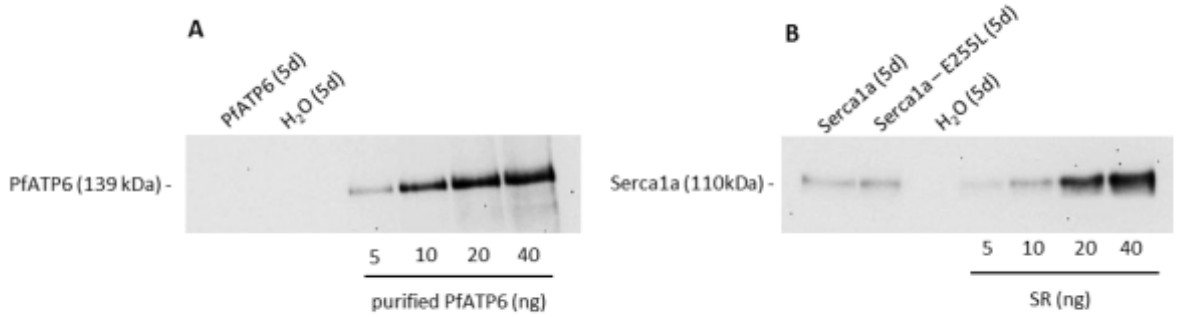


Figure S4 - Expression profiles of PfATP6, SERCA1a and SERCA1a–E255L in microsomal membranes (P3) of oocytes. *Xenopus laevis* oocytes were injected with water as control and mRNA for PfATP6 (A), SERCA1a or SERCA1a–E255L(B). The P3 oocyte membranes were prepared. Each lane represents 1 µg of total final protein. 5d – 5 days. Purified PfATP6 is derived from yeast expression.

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III.3 – Conclusions

With this paper we were able to show that the mutant SERCA_E255L, expressed in *X. laevis* oocytes was not sensitive to artemisinin. We were not able to express an active form of PfATP6 in these oocytes, hence the effect of artemisinin upon the Ca^{2+} - dependent ATPase activity could not be measured. Nevertheless, based on the results on SERCA1a and SERCA1a_E255L we can affirm that Krishna's and coworker's results cannot be reproduced. This work can therefore put an end to the so long established controversy on Krishna's and collaborator's findings on *X. laevis* oocytes.

Perspectives and Conclusion

IV – Perspectives

IV.I – Perspectives on PfATP6 Studies

The large scale production of PfATP6 brought a new dimension to the project. With the established protocol we are able to purify a sufficient amount of PfATP6 for compound screening. The implementation of a microplate ATPase activity measurement protocol, that uses less protein, opens the possibility to test several molecules. Thus it is interesting to maintain and create new collaborations in view of finding new PfATP6 inhibitors.

The modification of the protease cleavage site (from thrombin to TEV proteases) for elution and purification of PfATP6 will enable a significant reduction of the purification costs and hence allow higher amounts of PfATP6 to be prepared, compatible with a screening of a larger library of molecules. These preliminary results showed us that substituting the cleavage site is not that trivial, and dealing with *Plasmodium* genes increases the challenge. In order to choose which are the best conditions to use a TEV cleavage site for the purification of PfATP6 the plasmid construction with the proper modification of BAD and TEV site to N-terminal of PfATP6, as well as testing lower concentrations of TEV and a NiTED resin to remove this protease, still have to be done.

A good starting point for the search of new PfATP6 inhibitors would be to test already validated molecules by MMV from the "Malaria Box". If one of these compounds reveals to be a PfATP6 inhibitor we would be in the good direction of finding the biological target of a compound. This would be very important information for the discovery and implementation of new antimalarial drug. Other than the importance of knowing the drug target for commercial reasons, it is also important to predict and counter possible resistance emergence.

For the best compounds issued from these studies it would be interesting to determine the erythrocytic stage upon which they act. For this it is important to have the previous knowledge of PfATP6 stage expression. We decided to investigate this by using immunofluorescence technique (in collaboration with Pr. Isabelle Florent and Dr. Lotfi Bounaadja). Unfortunately, after several assays and attempts in optimizing this protocol, we were not successful in visualizing a specific staining. We think this might be due to protocol troubleshooting (blocking or washing steps, or even permeabilization steps) or maybe due to unspecific staining of the fluorofore used (green fluorescent Alexa® Fluor 488 probe). We observed, with another protein, that the Texas-Red® dye was much more specific, using the exact same protocol. This dye may be attempted for immunofluorescence technique on PfATP6 but also other techniques such as mRNA detection can be performed. After the determination of the stage-specific expression of PfATP6, the compounds can be tested on stage-synchronized *P. falciparum* cultures.

For the more profound knowledge of PfATP6 and its role in calcium transport in *P. falciparum,* it would be interesting to reconstitute this protein in proteoliposomes. This could of course be used as a direct activity test, by measurement of calcium transport. But this knowledge could also give more insight into this mechanism in an Apicomplexan organism, and enlighten about an ortholog feature from a phylogenetically distant organism from mammals. Maybe mutants of this protein could give some answers about the role of the large cytosolic loop that is absent in mammalian SERCA1a (see

Figure 20 of the Introduction section). These reconstruction have been previously attempted by D. Cardi during her PhD thesis (Cardi, 2009) but with no success, a calcium uptake was not possible to measure, maybe due to a lack of stability of PfATP6.

The crystallization project of PfATP6 has been started when this protein was purified. Several conditions for PfATP6 crystallization, based on past experience with SERCA1a, have been attempted previously by Delphine Cardi, Bertrand Arnou, but also as part of this PhD project. Unfortunately with no success. The previously mentioned loop (see Figure 20 of the Introduction section). would probably be too unstable for crystallization. Some strategies have been formulated:

1. Construct a *pfatp6* – *serca1a* chimera gene where the PfATP6 loop sequence would be replaced by the SERCA1a sequence.

2. Construct a *pfatp6* gene lacking poly-asparagine sequences that are known to be unstable for crystallization. This can be achieved either by molecular biology constructions or by specific proteolytic enzymes that recognize and cleave asparagine residues by the Tsh autotransporter from *Escherichia coli* (Rawlings et al., 2011)

3. And use specific PfATP6 inhibitors to block the protein. This would need, for instance, to perform phosphorylation experiments to determine on which part of the cycle each identified PfATP6 inhibitor blocks the protein.

The crystallization of PfATP6 would be an important achievement for general knowledge of PfATP6 as few *P. falciparum* transporters are described and additionaly giving insight into another SERCA protein, this time from an Apicomplexan organism; but also to design new inhibitors and explore this transporter as a potential antimalarial target.

As PfATP6 has been chemically (Arnou et al., 2011; Cardi et al., 2010b; David-Bosne et al., 2013) and genetically (Pulcini et al., 2013) validated as an antimalarial target, all of these achievements in establishing new solid collaborations and protocols for PfATP6 inhibitors searching, are of great interest for antimalarial research.

IV.2 – Perspectives on PfAdT Studies

PfAdT was considered one of the most promising antimalarial targets to explore (Staines et al., 2010).

PfAdT was expressed in yeast *S. cerevisiae* and in bacteria *E. coli.* These two organisms were used for different purposes. Yeast will enable the purification of this protein and *E. coli* will be useful for functional studies and inhibitors screening. Thus it would be interesting to further continue this project in the following lines:

- Optimize a purification protocol for PfAdT_wt and PfAdT_K24I expressed in yeast, from the knowledge we already possess for purification of human ADP/ATP carrier (hAAC).

- The purification of PfAdT will enable structural studies of this carrier, as performed before with the bovine AAC (bAAC) (Dahout-Gonzalez et al., 2003). We can hope that the crystallization conditions could inspire an attempt to crystallize PfAdT, as the two proteins present 60% of identity. This will bring important structural information about this promising carrier, but also for further inhibitors design that will exploit the differences between the human and the parasite ADP/ATP carrier. On the other hand, the crystallization of both PfAdT wild-type and the mutant K24I could bring interesting knowledge about this inactive mutant and the function of this protein.

- PfAdT_wt and PfAdT_K24I can always be reconstructed into a proteoliposome for functional studies, but also drug screening as described for transport proteins of other pathogens such as achieved with *Pseudomonas aeruginosa* (Verchère et al., 2014). This procedure has already been tried in the laboratory for the hAAC but with no success. This may be due to ATP leaking from non intact proteoliposome. In the future, to investigate if these are intact, electron microscopy technique could be performed to visualize the reconstituted protein vesicles. Another reason might be the 50% orientation of the carrier towards the interior or the exterior of the vesicle. As we will be observing an ATP transport, the ATP that entered the vesicle could be rapidly transported again to the exterior, by a transporter oriented in the opposite direction, and no effect will be observed. Unlikely what is observed with ATPase membrane transporters that, even though 50% of the pumps are oriented in the opposite direction, the ATP needed for the transport is available only in the exterior. Hence, only the pumps oriented in the right direction will be activated. A way to overcome this challenge for the ADP/ATP carrier is to apply an inhibitor in only one side of the compartment to inhibit transport. Although one has to be careful that this inhibitor will block only one of the orientations of the proteins, and not to have affinity to an exposed part common to both orientations. Bongrekik acid might fullfil this requirement (Razakantoanina et al., 2008).

- After the expression of PfAdT_wt and PfAdT_K24I in *E.coli,* an ATP transport protocol can be optimized and adapted from previous studies (Razakantoanina et al., 2008). Radiolabeled ATP uptake can be measured and the procedure optimization can be first performed with AAC classical inhibitors (bongkrekic acid and carboxyatractyloside).

- A first functional test can be performed in order to determine if these proteins are active and try to determine an activity. Then we could study the ATP transport differences between PfAdT_wt and the supposedly inactive mutant PfAdT_K24I. This mutant, if functionally inactive as previously observed with bAAC (Ravaud et al., 2012), will constitute a good negative control for functional studies.

- It will be interesting to give more insight into the function of this protein and ADP/ATP transport. This K24I mutant can bring important answers of this translocase but also of ADP/ATP transport in a more distant organism than the ones already described.

- In a second phase, the Malaria Box from MMV can be tested upon PfAdT_wt. If some of these already known antiplasmodial molecules prove to be inhibitors of PfAdT, we might have found the target of thess compounds. This will enable design of derivatives of these compounds in order to find a suitable antimalarial.

- In parallel it will be of great importance to start collaboration with chemists for inhibitors design for PfAdT but also PfATP6. Homology structural models can be constructed based on the structures of bAAC and hAAC and homology of sequences with PfAdT. This will be a good starting point for inhibitors design.

- Finally one can always screen for inhibitors from a chemical library in hope to find specific hits that do not inhibit hAAC.

- In the future the best compound hits will have to be tested for the effect on the *in vitro* growth of *P. falciparum* and their cytotoxicity on mammalian cells. If ever a compound meets MMV requirements, it will be therefore needed to be tested *in vivo* on a rodent model for malaria, on *P. vivax in vitro* cultures, on multidrug resistant *P. falciparum* strains and for neurologic, cardiologic, reproductive toxicity and mutagenesis before entering clinical trials phases.

With this project still in its initial phase, there is so much to be discovered and studied in the field of *P. falciparum* ADP/ATP transport. This will give new insight into a new *P. falciparum* mitochondrial transporter that might be a good potential antimalarial target if one can manage to exploit the differences between the human and the *Plasmodium* carrier. A lot remains to be done with this protein, which can become a very enthusiastic and interesting project to continue.

V - Final Conclusions

The subject of this PhD thesis was the study of *Plasmodium falciparum* transporters as potential drug targets. We have chosen three candidate transporters to be studied: PfATP6 and PfAdT.

PfATP6 is the *P. falciparum* SERCA and has been subject of a longstanding controversy as it was described to be the direct target of the most widely used antimalarial – artemisinin. The laboratory, in which this PhD has been achieved, has a well established expertise in SERCA1a protein from rabbit muscle. This expertise and the interest that arose around PfATP6 led the team to start the study of this protein. The yeast expression and purification by affinity-chromatography protocols were first established during D. Cardi's PhD thesis (Cardi, 2009) and demonstrated that purified PfATP6 was active but not inhibited by artemisinins (Cardi et al., 2010b). Due to the biological role of SERCA pumps and the homology between PfATP6 and SERCA1a, this transporter was established as a potentially interesting antimalarial target (Arnou et al., 2011).

With this PhD project we intended to explore PfATP6 as an antimalarial target. We first established an optimized protocol for large-scale purification of PfATP6 and an ATPase activity test in 96-well microplates. From this, collaborative projects were established and enabled screening of compounds from a P-type ATPase chemical library. We indentified new PfATP6 inhibitors, and tested these inhibitors for their antiplasmodial activity and mammalian cytotoxicity. We also tested molecules with known antiplasmodial activity (4-aminoquinoline-clotrimazole based compounds). We were

able to validate chemically this target (David-Bosne et al., 2013), that was also genetically validated (Pulcini et al., 2013).

PfAdT is the adenylate translocase of *P. falciparum* and was described as being a promising potential antimalarial target (Staines et al., 2010). The laboratory had a collaboration for the production of hAAC in yeast and purification for functional and structural studies, hence we aimed to use this knowledge to express PfAdT in yeast for further purification. However, for functional studies and compound screening on PfAdT, we decided to adopt the methodology described by Razakantoanina et al 2008, and express this protein at the plasma membrane of C43 (DE3) *E. coli* strain (Miroux and Walker, 1996).

Malaria Medicine Venture (MMV) has an available library of about 400 molecules, described to possess a potent antiplasmodial activity, but no knowledge regarding the molecular target. This so called “Malaria Box”, is freely available from their website for research purposes. It will be of great interest to test some of these molecules in a first attempt of finding new PfATP6 and PfAdT inhibitors, while new collaborations with chemists are being contemplated.

In the future, the establishment of PfAdT purification protocol and the already well established PfATP6 purification procedure will enable an investment in a crystallization project for these transporters. This will be of great interest for inhibitors design but also as structural knowledge about so poorly known *P. falciparum* transporters.

The work done on these transporters allies knowledge of biochemical tools for membrane protein expression and purification along with malaria biological expertise, in the field of antimalarial research.

Material and Methods

I – DNA Vector Construction

I.1 – Yeast Expression Vector Construction

The gene of interest was cloned into a pYeDp60 vector that contains a part for amplification in bacteria with an origin of replication (Ori c) and an Ampicillin resistance gene; a part for yeast vector replication with two auxothrophy markers for selection of positively transformed yeast (adenine and uracil); and the gene of interest under the control of a strong promoter *CYC1* (iso-1-cytochrome c), *GAL10* inducible (Cardi et al., 2010a) (*Figure 54*).

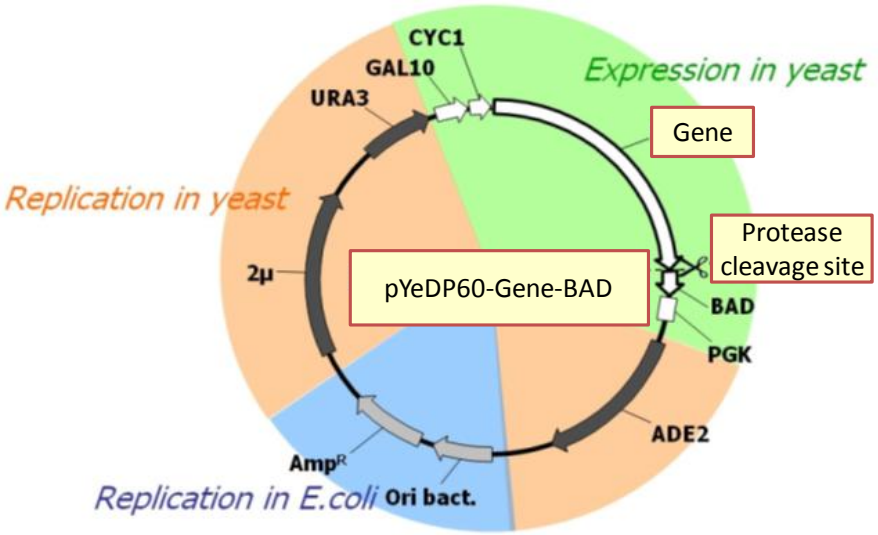

Figure 54 - Yeast expression vector pYeDp60 construction with the gene of interest.

Cloning and amplification in Escherichia coli (light grey): Ori bact - bacterial replication origin; Amp ^{R} - gene coding for β -lactamase to allow resistance to ampicillin (selection marker). Amplification in yeast (dark grey): ADE2 - auxotrophy selection marker for adenine; URA3 - auxotrophy selection marker for uracil; 2µ - yeast replication origin. Expression (white): GAL10-CYC1 - fusion promoter of the inducible part of GAL10 and RNA polymerase binding part of CYC1; PGK - phosphoglycerate kinase terminator sequence; Gene coding sequence; Protease cleavage site - sequence coding for a protease cleavage site; BAD - biotin acceptor domain. The Gene, protease cleavage, and BAD sequences were cloned in the same coding frame to allow expression of the fusion protein-BAD.

PfATP6 was cloned into pYeDp60 according to the procedure described in (Cardi et al., 2010a), with a BAD domain (Biotin Acceptor domain) that will express at C-terminal of the protein, preceded by a thrombin or a TEV cleavage site, the later recently constructed by Christine Jaxel and Benoit Jacquot. To substitute the thrombin cleavage site for a TEV cleavage site, the gene was first amplified by PCR (primers in Table *12*), and using EcoRI and NotI restriction enzymes, the gene was inserted in pYeDp60 vector.

PfAdT was cloned into the pYeDp60 vector, with a His_6-Tag and the BAD domain at the N-terminal end of the final protein, followed by a TEV cleavage site. The cloning was performed from the *E. coli* expression vectors PfAdT– pET14b (given by I. Florent). A first amplification by PCR with the primers described in Table *12* was performed. The insertion in pYeDp60 was achieved using Sal I and Kpn I restriction enzymes (New England – Biolabs).

I.2 – E. coli Expression Vector Construction

For functional studies, the gene *PfAdT* was inserted in a pET20b vector given by Stéphanie Ravaud, fusioned to a Maltose binding protein sequence (MBP) at the N-terminal end of the final protein, followed by a thrombin cleavage site. MBP is important to export the final protein to *E. coli's* periplasm in order that PfAdT expresses at the bacteria plasma membrane (Razakantoanina et al., 2008). A PCR from *PfAdT* - pET14b DNA given by Isabelle Florent, was performed to amplify the gene using the primers described in Table *12*. The insertion of the *PfAdT* gene was achieved with Xma I and Xho I restriction enzymes (New England – Biolabs) (see below for experimental procedure). The mutated form of *PfAdT* was obtained by direct mutagenesis by PCR amplification and replication of the vector in *E. coli*, after ligation of the PCR product (T4 DNA ligase – New England Biolabs) (see below).

I.3 – Molecular Biology Techniques

I.3.1 - Polymerase chain reaction

PCR was performed on a thermocycler machine (G-storm, Ozyme) using 50µl of a PCR reaction mixture containing: 0.02 units/µl of High-fidelity DNA polymerase (Phusion, Finnzyme); 1X HF buffer (Finnzyme); 0.2mM of dNTPs; 0.25 pmol/µl of primer reverse and forward; and the DNA template (5 – 50 ng). PCR conditions : denaturation 98ºC for 1-2 min; primer annealing temperature chosen (see below) for 1 min; elongation 72ºC for 1–4 min, 30 cycles.

Prior to the amplification of DNA, we test our newly synthesized primers by a temperature gradient PCR as follows: denaturation 98ºC for 1-2 min; primer annealing gradient temperature 50 ºC–70ºC for 1min; elongation 72ºC for 1–4 min; 30 cycles. PCR products are purified with a G100 column (Corre et al., 1997), and are verified by 1% standard agarose gel in TAE buffer (Tris-acetate pH8, EDTA). Migration is undertaken for 2h at 60V. The optimal annealing temperature for these primers is chosen for further PCR amplification. Though, if all PCR products are good, they are simply pooled and used as such.

The primers used were the following:

Cloning	Forward primer	Reverse primer
PfATP6 – TEV - BAD	5′-CTA AAT TAC CGA ATT CTA GTA TGG AAG AAG TTA TTA AAA ACG CTC-3′	5′-GCC CTG AAA ATA AAG ATT CTC CGC GGC CGC ACC ACC ATC AAT CTT AAT CTT TTT AGT-3′
BAD – TEV – *PfAdT* – pYeDp60	5'-CAC AGT CGA CTA TGA GTT CTG ATA TAA AAA CC-3'	5'-CAC AGG TAC CTT AAA TCA ATT TTT GTA ATT CAT C-3'
MBP – thrombin – *PfAdT* K24I – pET20b	5'-CAC ACC CGG GGC AGC GCG GCC GCA ATG AGT TCT GAT ATA AAA ACC-3'	5′-CAC ACT CGA GTT AAA CTA ATT TTT GTA ATT C-3'
MBP – thrombin – *PfAdT* wt – pET20b (directed mutagenesis)	5′-TCA GCC GCA ATA TCA AAA ACA GTG GTT GCT-3′	5′-AGC AAC CAC TGT TTT TGA TAT TGC GGC TGA-3′

Table 12 - Primers used for cloning purposes

I.3.2 - Directed mutagenesis

Direct mutagenesis was performed on *PfAdT* gene to perform the modification K24I (Figure 55). To achieve this we designed the above primers (Table *12*). The PCR reaction undergoes normally with the following conditions: denaturation 98ºC for 1-2 min; primers annealing temperature chosen for 1 min; elongation 72ºC for 4 min; 30 cycles. After purification and verification of the PCR products on a 1% agarose gel, they are ligated to form the vector (Figure 55).

I.3.3 - Ligation

PCR products and plasmids are digested with the chosen restriction enzymes, the DNA is ethanol precipitated, purified on 0.5 – 1% resophor agarose gel in TAE. The agarose slice is purified with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) and DNA is quantified by migration on a 1% standard agarose gel in TAE. The ligation of insert and vector are done with 1 µl T4 DNA ligase (New England Biolabs), 50 ng of vector and 3 times more insert. The ligation is done at 16ºC overnight.

Ethanol DNA precipitation – to the DNA is added: 1/3 volume of $AcONH_4$ 10M pH 5.2, 2.5X 100% Ethanol. The tube is incubated for 30 min at -20ºC and then centrifuged for 30 min at 13000 rpm and 4°C, the supernatant is removed. The pellet is washed (twice) with 1 ml of 70% ethanol (kept at -

20ºC), the tube is vortexed and centrifuged for 30 min at 13.000 rpm and 4°C, the supernatant is removed. Then, 100 µl of 100% ethanol (kept at -20ºC) is added, the tube is vortexed and centrifuged for 30 min at 13.000 rpm and 4°C. The supernatant is removed and the pellet is dried in a Vacuum Concentrator (SpeedVac ™, Thermo Scientific) and kept at 4ºC after being re-suspended in water before use.

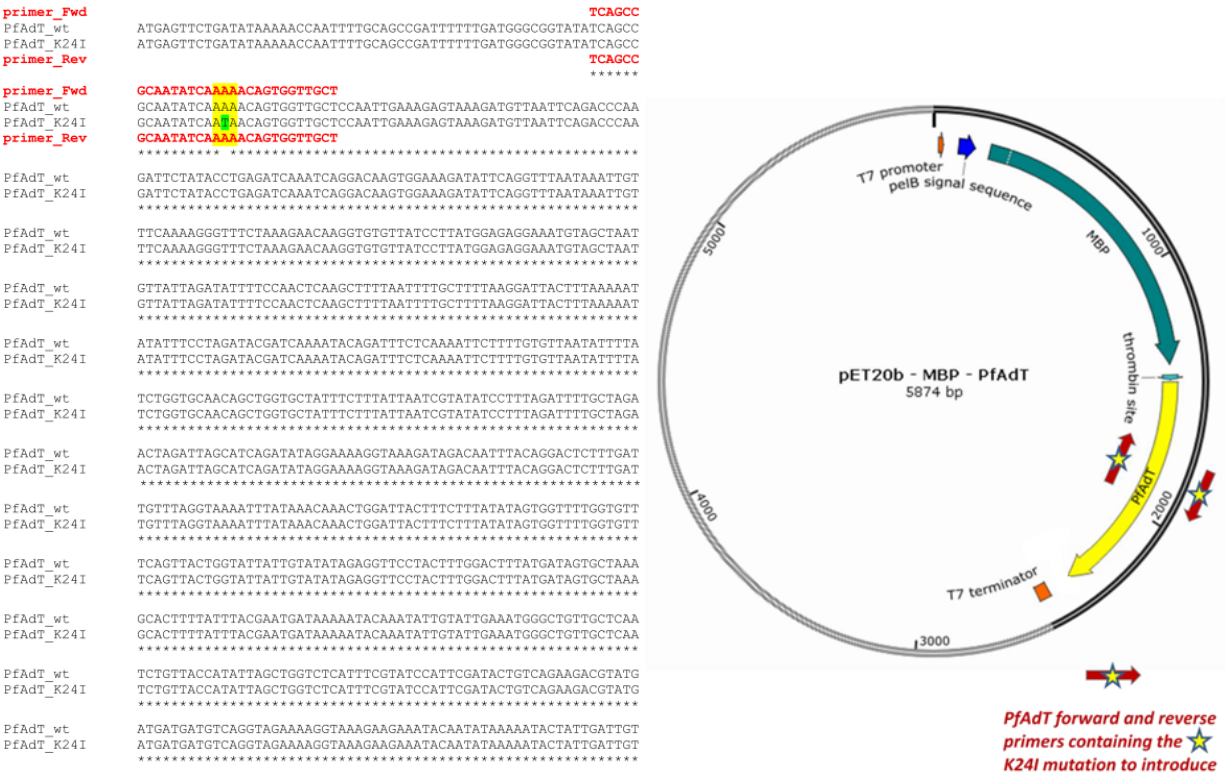

Figure 55 – Design of the direct mutagenesis strategy to obtain PfAdT wt and k24i form of the gene.

I.3.4 – Competent *E. coli* preparation

The preparation of JM109 or C43 (DE3) competent *E.coli* is done by previous thawing of the cells kept at -20ºC, and pre-culture in LB medium at 37ºC and 180 rpm. The day of the preparation, 1 ml of pre-

culture is taken and used to inoculate 100 ml of LB (pre-incubated at 37 ºC and 180 rpm). The culture proliferates until it reaches 0.3-0.6 OD_{600} (+/-2 hours). The culture is centrifuged for 5 min at 5000 rpm and 4 ºC (rotor JA 12, Beckman Coulter™, Avanti™ J-20XP). The supernatant is discarded and the cell pellet is gently re-suspended in 15 ml of buffer (50 mM $CaCl_2$ – 10 mM Tris pH 8). The cells are kept for 20 min on ice and then centrifuged for another 5 min at 5000 rpm and 4 ºC. The supernatant is discarded and cells are re-suspended in 1.5 ml of the same buffer and left on ice for 1 hour. The competent bacteria are ready to use or to quick freeze in liquid nitrogen and stored at -80ºC.

I.3.5 – Transformation

Competent *E. coli* cells (100 µl) are transformed with all of the ligation, 7.5µl of buffer (500 mM $CaCl_2$ – 50 mM Tris pH 8) and water *qs* 200 µl. The cells are left on ice for 30 minutes and heat shocked at 42ºC for 1min. Then 1 ml of LB medium is added and they are left proliferating for 1h at 37ºC and 180rpm. After they are pelleted for 5 min at 5000 rpm, the supernatant is removed and they are plated into a LB ampicillin – agar medium Petri-dish. The bacteria are left to proliferate overnight at 37ºC or for 2 days at room temperature.

Positive clones are checked, after growing overnight at 37 ºC and 180 rpm in liquid LB ampicillin medium and by performing Miniprep (Macherey-Nagel Kit). Minipreps are digested with restriction enzymes that cut either once or twice in the DNA construction, and ran on a 1% standard agarose gel.

I.3.6 - Sequencing

Before transforming yeast with the vector construction, this one is verified by Sanger sequencing method (eurofins – MWG operon), using the same primers used for PCR amplification of the insert, and if needed (gene > 1000 bp) with primers in the gene (Table 12).

II – Protein Expression, Purification and Activity Measurement

II.1 – Yeast Strain

For protein expression we used *Saccharomyces cerevisae* strain W303.1b/Gal4_2 (*a*, *leu2*, *his3, trp1*::*TRP1-GAL10-GAL4, ura3, ade2-1, can*^{r}, *cir*$^{+}$) previously described (Lenoir et al., 2002). This strain has the particularity to be of matting type *a;* to be auxothrophe for amino-acids leucin and histidine, and for nitrogen bases uracil and adenine that can be used for selection; to possess the *Gal4* gene; as well as sensitivity to canavanine and to contain a 2µ plasmid.

Gal4p is a positive regulator of the transcription of *GALl10* that is responsible for coding for an enzyme used to convert galactose into glucose. In presence of glucose this transcription factor is repressed. When glucose is completely consumed and galactose is added, Gal4p is released and activates the *GAL10* promoter allowing high synthesis of the transactivator and then inducing the transcription of the gene under the promoter, for instance *PfATP6* (Cardi et al., 2010a).

II.2 - Yeast Culture Media

All culture media products were purchased from Becton Dickinson Bioscences (Gibco BRL, Fisher Scientific Bioblock). All chemical products were acquired from Sigma-Aldrich, except when mentioned otherwise.

All media are sterilized for 20 min at 120 ºC. Glucose and galactose, adenine and ethanol are added only after sterilization and cooling of the medium from, respectively, a glucose or galactose 40% stock solution, an adenine 10mg/ml stock solution, and an ethanol 96% (v/v) commercial solution.

YPD (A) medium: 1% (w/v) bactopeptone, 1% (w/v) yeast extract, 1% (w/v) glucose, 20µg/ml adenine.

YPGE (2X) medium: 2% (w/v) bactopeptone, 2% (w/v) yeast extract, 1% (w/v) glucose, 2.7% (v/v) ethanol.

S6 (A) medium: 0.1% (w/v) bactocasamino acids, 0.7% (w/v) yeast nitrogen base, 2% (w/v) glucose, 20µg/ml adenine.

S6 (A) agar medium: 0.1% (w/v) bactocasamino acids, 0.7% (w/v) yeast nitrogen base, 1.5% agar, 2% (w/v) glucose, 20µg/ml adenine.

S5 (A) medium: 0.1% (w/v) bactocasamino acids, 0.7% (w/v) yeast nitrogen base, 2% (w/v) galactose, 20µg/ml adenine.

40% (w/v) glucose and galactose stock solution are sterilized by filtration onto a 0.22 µm Steritop filter (Milipore S.A.)

10 mg/ml adenine solution is prepared in 0.1*N* HCl and filtrated onto a 0.22 µm Steritop filter (Milipore S.A.)

Plate buffer: 40% polyethylene glycol (PEG) 4000, 100 mM Lithium acetate, 20 mM tris-HCl pH 7.5, 2 mM EDTA.

II.3 - Yeast Transformation

The yeast strain above described was used to transform the vector construction containing the gene of the protein of interest. The yeast strain is maintained on Petri dishes for < 1 month at 4°C in an YPD (A) – agar medium. An over-night pre-culture of the W303.1b/Gal4_2 strain is undertaken in 5ml of rich YPD (A) medium, at 28°C and 140 rpm in an shaking incubator. In the morning of the second day, the Optical Density at 600 nm (OD_{600}) of the pre-culture is taken and is normally around 1 – 2 OD_{600}. The yeast are diluted to a final density of 0.1 OD_{600}, in 10 ml of preheated to 28°C YPD (A) medium and incubated at 28°C and 180 rpm. When the OD_{600} reaches between 0.3 – 0.5 OD_{600} ($3x10^7$ – $5x10^7$ cells per ml) they are in the exponential growth phase. The volume corresponding to $5x10^7$ cells (or 0.5 OD_{600}) is taken into a sterile tube. Cells are centrifuged at 5000 rpm (rotor FX301.5, Beckman Coulter™ - Allegra X-30R Centrifuge) for 5 minutes. The supernatant is eliminated and 50 µl of denaturated Salmon Sperm DNA (SSD) (2 mg/mL) by heating for 5 minutes at 100°C, and 1µg of plasmid are added (as a negative control an equal volume of water is added instead of the DNA). After vortexing, 500 µl of plate buffer and 20 µl of dithiothreitol (DTT) 1M are added. After vortexing, the tubes are left overnight at room temperature. The morning of the third day the tubes are centrifuged at 2000 rpm for 2 minutes. 100 µl of S6 (A) medium is added to each pellet. After the pellets are re-suspended, the yeast are plated into an S6 (A) – agar medium on a petri dish and left at 28°C during 2 days. The selection of positive clones is done by uracil deprivation.

II.3.1- Verification of the transformation

II.2.3.1- Verification of the transformation by expression in minimum medium

After transforming yeast with the vector construction we need to verify the transformed clones, *i.e.*, that they incorporated the good plasmid and that they are able to express in sufficient amount the protein we aim to produce. This way we may choose the best clone to cultivate in larger quantities in rich medium.

A pre-culture of normally 4 distinct clones is performed in 10 ml of S6 (A) medium at 28°C and 160 rpm overnight. In the morning of the second day the OD_{600} of the pre-culture is verified and should be between 1 and 2 OD_{600}. We keep each one of these clones on an S6 (A) – agar Petri dish and only then we proceed to the inoculation of 20 ml of S5 (A) medium at a final OD_{600} of 0.2. These are incubated at 28°C and 160 rpm for 17h. The S5 (A) medium contains galactose that will induce the expression of our protein, under the Gal10 inducible promoter. On the third day the OD is measured and an equal amount of cells is taken (usually, a final amount corresponding to a total of 10 OD_{600}) and centrifuged at 1000 *xg* at 4°C for 10 minutes. The supernatant is discarded and the pellet is then washed in 1 ml of TEPI buffer (50 mM tris-HCl pH 6.8, 5 mM EDTA, 20 mM NaN_3, 1 mM PMSF, 1 X complete EDTA-free protease inhibitor cocktail tablets from Roche). Another centrifugation of 3500 rpm is performed at 4°C for 10 minutes. The supernatant is discarded and the pellet is re-suspended in 100 µl of TEPI buffer. An equal amount of glass beads (0.5 mm diameter, Biospec Products) are added. Yeast are broken for 20 min at 4°C on a *vibrax*. The supernatant is transferred to an ultracentrifuge tube of 1.5 ml. Beads are washed with 100 µl of TEPI buffer and the buffer is added to the ultracentrifuge tube. The samples are ultracentrifuged at 40 000 rpm for 1h30 at 4°C in a rotor TLA 45 (Beckman Coulter™ TL-100 Ultracentrifuge). The supernatant is discarded and the pellets are quick frozen in liquid nitrogen and then kept at -20°C. This protocol is always performed at 4°C.

The day the western blots are performed (section *II.9.3*), the pellets are re-suspended in 100 µl of D2XU (100 mM tris-HCl pH 6.8, 9 M urea, 1 mM EDTA, 5% SDS, 0.01% bromophenol blue, 5% 2-mercaptoethanol).

II.2.3.2 - Verification of the transformation by expression in rich medium

The protocol in section *II.4.1 –Culture in Fernbach Flasks* is followed, although the final volume of YPD (A) is 120 ml (instead of 500 ml) and the rotor used for centrifugation is SX-4400 rotor (instead of JLA 8.1000).

For verification of the expression, we do a membrane preparation from the harvested yeast, as described in section *II.5 - Membrane preparation*, with adaptation of the volumes.

The expressed proteins are detected by Western Blot or Coomassie blue staining (see below).

II.4- Expression of PfATP6 and PfAdT in Yeast

Once the positively transformed clones are selected and verified to be able to express our protein, we proceed to the culture of the yeast at high density and in larger quantities in rich medium. The culture can be performed in Fernbach flasks using a common shaking incubator, to culture from 500 ml and up to several liters of culture; or can be cultured at high density from 6 L up to 20 L in a bioreactor (Techfors-S from INFORS – HT coupled to a recirculating cryostat FE1100 from Julabo). The advantages of using the bioreactor are that the culture can be grown at higher density, in a

homogenous way, and is almost completely monitored by the machine. The Fernbach flasks require a human presence to induce expression, to cool and to stop the culture. It also introduce variability between flasks. However, it also enable to culture smaller volumes and hence to test specific conditions. The growth conditions for expression of the Ca^{2+}-ATPase were undertaken as previously described (Cardi et al., 2010a; Centeno et al., 1994; Lenoir et al., 2002).

II.4.1 - Culture in Fernbach Flasks

On day 1 the selected clones are pre-cultured in 5 ml of S6 (A) for 24h at 28°C and 180 rpm. On the second day the OD_{600} must be around 2; 50 ml of S6 (A) medium are inoculated at a final OD_{600} of 0.1 and left incubating at 28°C and 180 rpm for 24h. The same day 500 ml of YPGE 2X medium is prepared into Fernbach flasks and sterilized. The media are incubated at 28°C and 130 rpm overnight. On day 3 the 500 ml of YPGE 2X are inoculated with the pre-culture at 0.05 OD_{600} and incubated at 28°C and 130 rpm during 36 hours. This growth phase is ended by cooling the cultures for 10 minutes on ice. The first expression induction is done (36h after inoculation) by adding 20 g/L of galactose powder and the flasks are incubated at 18°C and 130 rpm for 13 hours. A second induction is performed (49h after inoculation) by adding 20 g/L of galactose powder and the flasks are incubated at 18°C and 130 rpm for 5 more hours. After 54 h the culture is stopped by cooling the flasks on ice for 10 minutes.

Yeast are harvested by 10 minutes centrifugation at 4°C and 4000 x*g* (JLA 8.1000 rotor, Beckman Coulter™, Avanti™ J-20XP). From this point the protocol is performed at 4°C. The supernatant is discarded and the yeast pellet is weighted and washed with 5 yeast volumes (w/v) of cold Milli-Q water. A second centrifugation is performed for 10 minutes at 4°C and 4000 x*g* (JLA 8.1000 rotor, Beckman Coulter™, Avanti™ J-20XP). The supernatant is discarded and the pellet is re-suspended in 2 yeast volumes (w/v) of TEKS buffer (50mM tris-HCl pH 7.5, 1mM EDTA, 0.6M sorbitol, 0.1M KCl) and incubated for 15 minutes at 4°C, to weaken the yeast membranes. Then the yeast are transferred into a disposable liner and centrifuged for 10 minutes at 4°C and 4000 x*g* (JLA 8.1000 rotor, Beckman Coulter™, Avanti™ J-20XP). The supernatant is discarded and the yeast pellet are quick frozen in liquid nitrogen and stored at -80°C.

II.4.2 - Culture in a Bioreactor

Growth of yeast cells and large scale expression of PfATP6 for inhibitors screening was performed using a bioreactor (*Techfors-S Apparatus, INFORS HT, Massy, France*) — The method was previously described in (Cardi et al., 2010a).

The 6 – 20 L of YPGE 2X medium (without glucose or ethanol) are directly prepared into the bioreactor and the sterilization program is launched. After cooling and before inoculating, the pH, oxygen and temperature probes are equilibrated. The culture medium is saturated with air by high aeration (1 volume of air per volume of medium per minute – 1 vvm) and agitation at 300 rpm for 12h.

The protocol described is for a 6L culture but can be easily adapted to a 20 L culture:

On day 1 (usually a Thursday) we do a pre-culture of the selected clone in 5 ml of S6 (A) for 24h at 28°C and 180 rpm. On the second day (usually a Friday) the OD_{600} must be around 2. We then do serial dilution in 5 ml of S6 (A) medium (dilution 1:100; 1:1 000, 1:10 000) that are incubated at 28°C and 180 rpm all the weekend (2 days). On day 5 (Monday) the most diluted culture (1:10 000) should be at an OD_{600} around 2. A dilution to 0.1 OD_{600} is done in two Erlenmeyer flasks containing each 60 ml of S6 (A) medium and are incubated for 24h at 28°C and 180 rpm. When the OD_{600} reaches 0.6 (exponential growth phase) the bioreactor may be inoculated. Before inoculation 1% glucose, 2.7% ethanol and 0.01% of foam suppressor (Antifoam A) are added to the growth medium. The medium is inoculated at a final density of 0.03 OD_{600} and the culture undergoes at 300 rpm and 28°C. the oxygen quantity is ideally set at 20% of dissolved dioxygen, and is regulated by the stirring conditions from 300 to 1000 rpm and air flow between 6 and 18 L/min (*i.e.,* 1 – 3 vvm in a 6 L culture), according to the needs in providing more or less oxygen to the culture. After 34h after inoculation, the first induction is prepared by lowering the temperature to 18°C (to slower yeast metabolism) and the stirring to 300 rpm as well as the aeration to 2 L/min. After 36h post-inoculation, the first induction of the expression of our protein is executed during 13h, by adding 20 g/L of galactose from a fresh 40% stock solution. After 49h post-inoculation, a second induction is triggered by adding 20 g/L of galactose and is followed during 5 more hours. After 54h post-inoculation, the culture is stopped and the temperature is decreased to 6°C, although the stirring is maintained to avoid yeast sedimentation (see Figure 56 for the following of yeast growth during culture in a bioreactor).

Yeasts are harvested as described above for culture in Fernbach Flasks (section II.4.1).

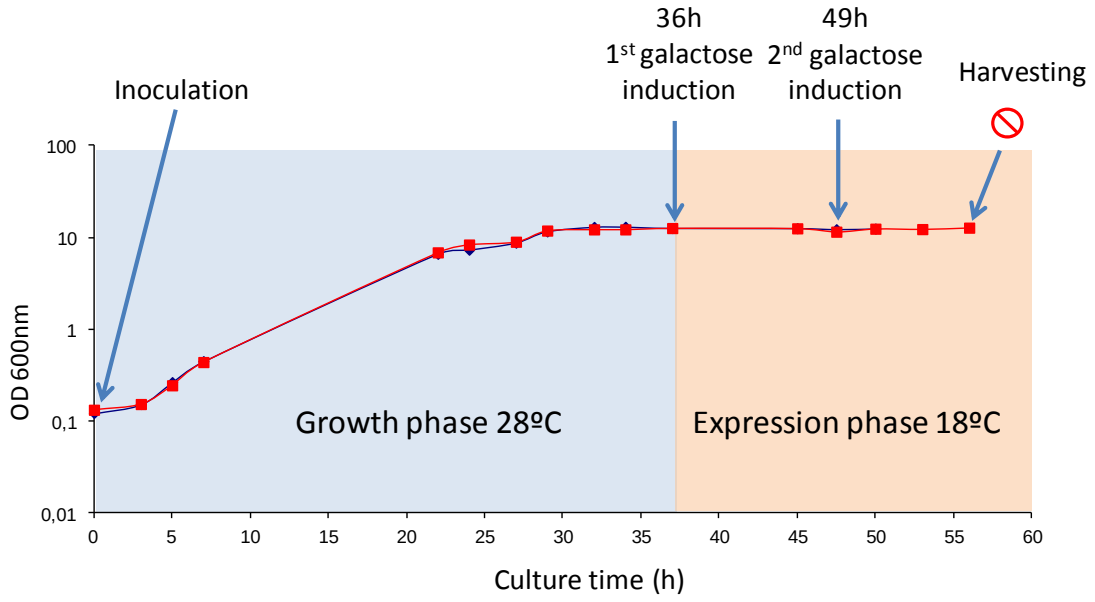

Figure 56 - Yeast growth following in a bioreactor.

Cell growth is measured by optical density at 600nm (OD_{600nm}).

II.5 - Membrane Preparation

The yeasts are unfrozen in a 20°C water bath and then placed in a cold room at 4°C where all the protocol is performed. The yeasts are re-suspended in equal volume (1 volume of yeast pellet) of cold TES buffer (50mM tris pH 7.5, 1mM EDTA, 0.6M sorbitol) supplemented with 2 mM of PMSF (phenylmethanesulfonylfluoride from a 200 mM stock solution in 2-propanol) and 2 X complete EDTA-free protease inhibitor cocktail tablets (Roche). They are transferred into agate pots with 1 yeast volume of 0.5 mm diameter cold glass beads. Yeast are broken in a Planetary mill *Pulverisette* (Fritsch) with the following protocol: 3 minutes agitation at 450 rpm, 30 seconds pause, 3 minutes reverse agitation at 450rpm. The supernatant is recuperated with a 25 ml plastic pipette into a graduated cylinder. The beads are washed three times with 0.5 volume of TES supplemented with 1 mM of PMSF and 1 X complete EDTA-free protease inhibitor cocktail. The pH is verified to be at 7.5; otherwise it is adjusted with NaOH. We here obtain what we call the Crude Extract (CE).The volume is annotated and a sample taken.

We then proceed to light membrane preparation through differential centrifugation. The CE is centrifuged at 1500 $_{gmax}$ for 20 minutes at 4°C (rotor JA 10, Beckman Coulter™, Avanti™ J-20XP). The pellet 1 (P1) is discarded but after re-suspension, the volume is annotated and a sample is taken. The supernatant 1 (S1) is recuperated and the volume is annotated and a sample is kept before centrifugation at 18000 $_{gmax}$ for 20 minutes at 4°C (rotor JA 10, Beckman Coulter™, Avanti™ J-20XP). The pellet 2 (P2) is discarded after its re-suspension; the volume is annotated and a sample is taken. The supernatant 2 (S2) is kept after volume annotated and a sample taken. S2 is ultracentrifuged at 40 000 rpm for 1 hour at 10°C (rotor Type 45 Ti, Beckman Coulter™ - L8_M Ultracentrifuge). We obtain pellet 3 (P3), after discarding the supernatant 3 (S3) from which a sample was taken and the volume annotated. The P3 membranes are re-suspended in 0.2 yeast volume of HS (20 mM HEPES pH 7.5, 0.3 M sucrose, 0.1 mM $CaCl_2$). They are then homogenized by passing into a potter, a sample is taken and the volume annotated before quick freezing in liquid nitrogen and kept at -80°C.

II.6 - Estimation of Protein Quantity in P3 Membranes

To be able to calculate the optimal quantity of detergent and resin used for the purification of our membrane transporters expressed in yeast, we first proceed to a protein dosage test by BCA assay (bicinchoninic acid assay) (Smith et al., 1985).

We use 96 wells plates. We first dilute in TES Buffer 10 times each of our samples from the membrane preparation (CE, S1, P1, S2, P2, S3 and P3). We use 10 µl of this dilution at which we add 5µl of 10% SDS, 10µl of Milli Q water and put into a well of the 96-well plate.

Bovine Serum Albumin (BSA) is used as the protein of known concentration to estimate the protein concentration of our samples. We perform a range of serial dilutions in Milli Q water (80, 40, 20, 10, 5 and 0 µg) using commercial BSA (Biolabs) at a concentration of 10mg/ml. Each known protein

concentration sample is placed in a well of the plate and 5 µl of SDS and 10 µl of TES buffer are added.

The coloring solution BCA: $CuSO_4$ (50: 1) is prepared and 200 µl are added to each well. The protein samples and the coloring solutions are directly mixed in each well by 10 up and down using a 200 µl micropipette. The plate is incubated for 30 minutes at 37°C. Absorbances are red at 540 nm in a spectrophotometer. With the absorbances values of known concentrations of BSA we are able to trace a absorbances/concentration curve and determine a polynomial (order 2) trendline. The equation of this trendline will enable the determination of unknown concentration of our samples through their measured absorbances. Like this we estimate the quantity of total protein in our sample.

II. 7 - Estimation of the Quantity of PfATP6 / PfAdT Expressed in P3 Membranes

To estimate the quantity of our transporter expressed, we perform a Western Blot with a specific anti-body against our protein (report to Western Blot protocol in section *II.9.3*). We include a range of a protein of known concentration that is then compared to the expression profiles of a sample from the P3 membranes prepared. As we know the exact concentration of total proteins of our P3 membranes, determined by the BCA assay, we can easily calculate the percentage of expression, relatively to the total proteins in these P3 membranes.

II.8 - Batch Purification using BAD domain by streptavidin-Sepharose Chromatography

In the vector construction a sequence coding for a Biotin Acceptor Domain (BAD) was inserted in the same open reading frame as the gene coding for our protein, preceded by a protease cleavage sequence. For instance, expressed PfATP6 will have a BAD domain at the C-terminal end, preceded by a thrombin cleavage site. The BAD domain will enable the protein to bind to a biotin during the expression phase (Figure 40 of the Results and Discussion section).

II.8.1- Solubilization

Solubilization of the P3 membranes is a crucial step for membrane protein purification. It is important to use a non-denaturating detergent and above its critical mycellar concentration (CMC) (Figure 57). To solubilize PfATP6 we use mild detergent DDM (n-Dodecyl β-D-maltoside).

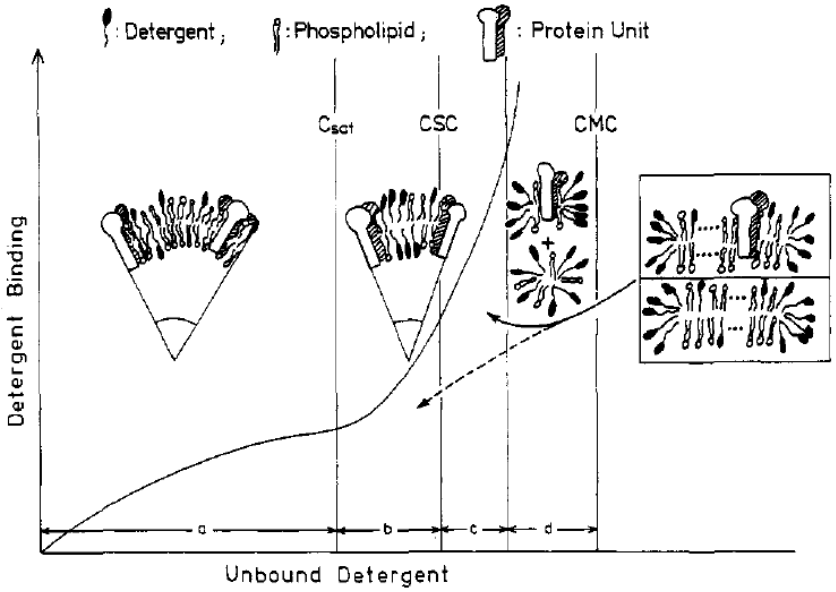

Figure 57 - ***Schematic representation of the various steps leading to the solubilization of membranar proteins, relatively to free detergent concentration.***

In phase a, detergent is non-cooperatively taken up by the lipid phase; in phase b, above C_{sat} *(concentration of free detergent demarcating the onset of cooperative binding of detergent by the membranes) detergent molecules cooperatively interact in the membrane, producing fragmentation as membrane sheets, but no solubilization of the vesicles; in phase c, at CSC (the critical solubilization concentration), lipid and protein units (monomers, protomers, or oligomers) start to become solubilized as small membrane sheets or bilayer-containing complexes, sealed at the edges by micellar detergent structures. In phase d, mixed lipid/detergent micelles and detergent-solubilized protein units, covered by any remaining lipid and detergent, are formed. Above the CMC (the critic micellar concentration), detergents organized as micelles and together with mixed micelles, pure detergent micelles are found in solution. Taken from* (Kragh-Hansen et al., 1993)*:*

P3 membranes are thawed in a 20°C water bath. The membranes are washed in pre-solubilization buffer (50 mM MOPS-Tris pH 7; 500 mM KCl, 20% glycerol (w/w), 1 mM $CaCl_2$, 1 mM PMSF, 1 X complete EDTA-free protease inhibitor cocktail, 1 mM 2-mercaptoethanol) at a final concentration 5 – 10 mg/ml to remove contaminant and soluble biotinylated proteins (Acetyl-CoA Carboxylase, Pyruvate Carboxylase and Arc1p). The diluted membranes are stirred for 30 minutes at 4°C (a sample TW is taken) and then ultracentrifuged for 1h at 40.000 rpm at 4°C (Type 45 Ti rotor, Beckman Coulter™ - L8_M Ultracentrifuge). The supernatant is discarded (sample SW) and the pellet is re-suspended at a protein concentration of 10 - 20 mg/mL in solubilization buffer (50 mM MOPS-Tris pH 7; 100 mM KCl, 20% glycerol (w/w), 1 mM $CaCl_2$, 1 mM PMSF, 1 X complete EDTA-free protease inhibitor cocktail, 1 mM 2-mercaptoethanol) with a Potter homogenizer, and put to stir in a beaker under magnetic agitation at 4°C. The detergent DDM is used at a ratio 1:3 (total protein in P3 membrane: detergent (w/w)) and diluted in equal volume of solubilization buffer used to dilute the washed P3 membranes. The DDM solution is added to the P3 membranes diluted little by little. When all the volume is added, the P3 membranes will be at a final concentration between 5 – 10

mg/ml of total proteins. Solubilization is performed during 1 hour under magnetic agitation at room temperature. After 30 min, the membranes were clarified by 10 to 20 up and down with a Potter homogenizer (sample TS). After, the solubilized membranes are centrifuged for 1h at 40.000 rpm (Type 45 Ti rotor, Beckman Coulter™ - L8_M Ultracentrifuge). The supernatant is kept to fix on the streptavidin-Sepharose resin, as it contains the solubilized membranes (sample SS).

II.8.2 - Fixation to the streptavidin resin

To purify our protein we use a streptavidin-Sepharose resin. As mentioned above, our protein has received a biotin during the expression phase thanks to the BAD domain coupled (Figure 40 of the Results and Discussion section).

The *Streptavidin-Sepharose TM High Performance* resin is used at a ratio of 1: 4 (v/w), using typically 4 mg of expressed protein per ml of resin, so we are at an excess of protein to be sure to saturate all available sites. The resin is previously washed in water and then solubilization buffer by several re-suspensions and centrifugations at 500_{gmax} for 5min, to remove the ethanol in which it is kept and equilibrate the resin in the right buffer. The supernatant after the centrifugation step was added to streptavidin-Sepharose resin and stirred gently overnight at 4°C (in the case of a thrombin protease cleavage), or for 2 h at room temperature (in the case of a TEV protease cleavage).

II.8.3 - Resin washing

After fixation, the resin is pelleted in 50 ml tubes for 10 minutes at 500_{gmax} and 4°C (rotor SX4400, Beckman Coulter™ - Allegra X-30R Centrifuge). The supernatant is eliminated (sample NR). The resin is washed two times in “high salt” buffer (50 mM MOPS-Tris (pH 7), 1 M KCl, 20% glycerol, 1 mM $CaCl_2$, 0.05% DDM, 1 mM 2-mercaptoethanol) (buffer: resin, 10:1 (v:v)) (sample HS). The resin is washed again two times in “low salt” buffer (50 mM MOPS-Tris (pH 7), 100 mM KCl, 20% glycerol, 1 mM $CaCl_2$, 0.05% DDM, 1 mM 2-mercaptoethanol) (sample HS), (buffer: resin, 10:1 (v:v)) (sample LS). The resin was re-suspended in the “low salt” buffer (buffer: resin, 1:1 (v:v)) (sample R).

II.8.4 - Protease cleavage

We used two types of cleavages, depending on the construction (Figure 55 and Figure 40 of the Results and Discussion section). Traditionally we used a thrombin protease (Cardi et al., 2010a), but we recently aimed to switch to a TEV protease cleavage to reduce the costs of the purification protocol, as we can produce this protease in *E. coli*.

For the thrombin cleavage: thrombin was added to the re-suspended resin at 10 units thrombin/ml of resin. The mixture was placed on a wheel and gently stirred at room temperature for 30 min (sample R30), followed by a second addition of thrombin and stirring for another 30 min (sample R60). To inactivate thrombin 1 mM PMSF was added during 5 minutes, and the solution of resin was transferred into Handee ^{TM} Centrifuge columns (Perbio Science France SAS, Brebieres, France).

For the TEV cleavage: TEV protease was added at 1: 10 dilution to the re-suspended resin. The mixture was placed on a wheel and gently stirred at 4°C overnight. The resin with the solution of cleaved protein was transferred into Handee ™ Centrifuge columns (Perbio Science France SAS, Brebieres, France).

TEV removal: as TEV is not inactivated by PMSF like thrombin, we need to remove this protease. The presence of a His_6-Tag, TEV protease enables the separation from the purified protein by passing the solution on a nickel affinity column (Ni-NTA).

II.8.5 - Elution

From the Handee ™ Centrifuge columns we can easily elute the proteolytically cleaved proteins. A first elution was undertaken (E1 sample) and then 1 resin volume of "low salt" buffer was added to re-suspend the resin and a second elution was performed (E2 sample). For the third elution, 1 resin volume of "low salt" buffer was added to re-suspend the resin, and the columns were centrifuged at 100_{gmax} for 10 min (E3 sample). The resin was kept until verification of the success of the purification by re-suspending it in 1 volume of "low salt" buffer and stored at 4°C (sample R*).

II.8.6 - Eluate concentration

The different elution fractions were pooled and concentrated on a Centriprep YM30 Centrifugal Filter Unit by successive centrifugations at 1500_{gav} (rotor JA-12, Beckman Coulter™ - Avanti™ J-20XP) for 30 min at 4°C until a final volume of 1-2 ml was obtained. The glycerol concentration was increased to 40 % before freezing the samples in liquid nitrogen and storage at -80°C.

The purification procedure is then verified by western blot (section *II.9.3*) with a specific antibody, and by Coomassie blue-staining SDS-PAGE gel, to quantify the amount of protein purified.

II.8.7 - Preparation of TEV protease

Prior to TEV preparation the clones are tested for their ability to express TEV. For this, a small culture of 5 ml of BL21 (DE3) *E. coli* is undertaken as described above, and after 2h of IPTG induction the equivalent of 0.5 final OD_{600nm} of bacteria is taken and centrifuged for 2 min at 10.000 rpm (rotor SX4400, Beckman-Coulter™ - Allegra X-30R Centrifuge). The supernatant is discarded and the pellet is re-suspended in 20 µl of Milli Q water and 20 µl of D2XU. The pellet is pre-heated at 95ºC for 1 min and then 8 µl are deposited on a 12% SDS-PAGE gel and revealed by Coomassie blue staining or Western Blot (see section *II.9*).

The coding sequence for TEV protease is inserted into an *E. coli* expression vector, with a C-terminal His_6-tag. Competent *E. coli* BL21 (DE3) cells are transformed with the vector (see sections I.3.4 and I.3.5) and pre-cultured in LB-ampicillin liquid medium at 37ºC and 200 rpm. Fresh LB ampicillin liquid medium is inoculated and incubated at 37ºC and 200 rpm until the culture attains an OD_{600} between 0.5 – 1 (exponential growth phase).The expression of TEV is induced by adding 0.5 mM of IPTG to the

medium and TEV expression is performed at 37ºC and 200 rpm for 3 hours. Culture is stopped and the bacteria are harvested by centrifugation at 5000 rpm for 10 min, at 10ºC. Bacteria are ressuspended in Tris-HCl 25 mM, NaCl 0.3 M, 20% glycerol, pH 7.5. then the bacteria are recentrifuged and ressuspended at a final concentration of 100 $_{OD600nm}$ / ml in Tris-HCl 25 mM, NaCl 0.3 M, 20% glycerol, 1 mM β—mercaptoethanol, pH 7.5 and kept on ice.

The resin Ni-NTA (Qiagen) is washed from the ethanol in which it is kept and it is left for 1 hour in buffer Tris-HCl 25 mM, NaCl 0.3 M, 20% glycerol, 1 mM β—mercaptoethanol, pH 7.5. In the mean time, bacteria are sonicated with a 10 mm Ø probe for 0.5 seconds pulse followed by a 0.5 sec stop, this during 1 min on ice. After sonication broken bacteria are centrifuged for 20 min at 10.000 rpm. The supernatant of the centrifugation is incubated for 1-2h with the resin at 4ºC under gentle stirring. The elution of TEV from the resin is performed with increasing amount of imidazole (9, 30 and 80 mM) and the elution is followed at 280nm. The eluates are concentrated on a Centricon® PM10 and the glycerol is resin until 50%. The samples are frozen in liquid nitrogen and kept at -80ºC. The estimation of the quantity of purified TEV is done by SDS-PAGE and Coomassie blue staining.

II.9 – *Protein Detection*

II.9.1 - SDS-PAGE gel

For SDS-PAGE, samples were mixed with an equal volume of denaturing buffer and loaded onto Laemmli-type 8% or 12% (w/v) polyacrylamide gels (Soulié et al., 1998). 8% polyacrylamide gels were used for detection of PFATP6 and 12% gels for detection of PfAdT. The amounts of proteins or volumes of initial samples loaded in each well are indicated in the figure legends. A molecular mass marker (Precision Protein Standards, Bio-Rad) was loaded in each gel. The migration of the samples on SDS-PAGE gels was performed for either 1h30 and 120V (8% gels for PfATP6 detection), either for 3h and 110V (12% gels for PfAdT detection), in order to obtain a good separation of the proteins present in each sample. Gels were stained either with Coomassie Blue, or proteins were electroblotted and transferred onto polyvinylidene difluoride Immobilon P membrane to perform Western Blot technique (Juul et al., 1995).

II.9.2 - Coomassie blue staining

After separation of the proteins on SDS-PAGE gels, the gels are stained for 30 minutes in a Coomassie blue solution (3mM Coomassie blue, 10% acetic acid, 45% ethanol). Then the gels are washed in Milli Q water. The excess of Coomassie blue is removed by several baths in hot Milli Q and the help of an absorbant paper. When the discoloration is satisfying the gels are dried between two sheets of cellophane and drying solution (10% ethanol, 10% acetic acid, 5% glycerol).

II.9.3 - Western Blot

After migration on SDS-PAGE gels of our samples we can also proceed to an immunodetection of our protein with a specific antibody. After electroblotting with CAPS buffer (10 mM CAPS, 10% Methanol, 0.1% NaOH 10N, Milli Q water *qs* 1L), membrane was blocked for 1 hour at room temperature (or overnight at 4ºC) in PBST (90 mMK_2HPO_4, 10 mM KH_2PO_4 (pH 7.7), 100 mM NaCl, 0.2% (v/v) Tween 20) containing either 5% of powdered skim milk, or 2% of BSA (depending on the antibody used, Table *13*). The primary antibody is then added to the solution (PBST-5% milk, PBST-2% BSA or just PBST, depending on the antibody Table *13*) and incubated for 1 h at room temperature. The membrane was washed three times 10 min with PBST and then incubated with the secondary antibody in PBST containing 5% powdered skim milk according to Table *13*. After three washes with PBST for 10 min each, detection of proteins was performed with ECL (GE Healthcare). The chemiluminescence signal was acquired with a GBox HR 16 apparatus coupled with GeneSnap acquisition software and analyzed with GeneTools analysis software (Syngene, Ozyme, France).

The antibodies were used at the following concentrations:

Detected proteins	**Primary antibody**	**Blocking solution**	**Concentration used**	**Secondary antibody**	**Concentration used**
PfATP6	Anti-PfATP6 polyclonal antibody (goat) (Cardi et al., 2010b)	PBST 5% milk	1:10.000 in PBST-5% milk	rabbit anti-goat IgG HRP	1:10.000 in PBST-5% milk
SERCA 1a	Anti-SERCA 79B polyclonal antibody (guinea-pig)	PBST – 5% milk	1:25.000 in PBST-5% milk	Rabbit anti-guinea pig IgG HRP	1:10.000 in PBST-5% milk
Biotinylated proteins	Avidin peroxidase (Sigma-Aldrich)	PBST – 5% milk	1:50.000 in PBST		
His-tagged proteins	His-probe - HRP (Thermo-Fisher Scientific)	PBST – 2% BSA	1:2.000 in PBST – 2% BSA		
MBP fusioned proteins	Monoclonal anti-MBP (New England Biolabs)	PBST – 5% milk	1:10.000 in PBST-5% milk		

Table 13 - Antibodies used for western blot technique, with the specific proteins they detect and the dilutions at which they were used.

II.10 –ATPase Activity Measurement

The measurement of the ATPase activity can be achieved by using a coupled enzyme system in a spectrophotometer 2 ml cell. This test is based on the oxydation of NADH to NAD^+. This reaction is proportional to the consumption of ATP by the active enzyme, as described in the Figure 32 of the Results and Discussion (East, 1994; Falson et al., 1997; Møller et al., 1980). For the reaction to take place we need to have the enzyme Pyuvate Kinase (PK) that with its substrate the PhosphoEnol Pyruvate (PEP) will renovate the quantity of ATP in the reaction mix, but also produce pyruvate that

is the substrate of the enzyme Lactate DesHydrogenase (LDH), which in turn produces lactate and leads to NADH oxydation. NADH quantity can be measured at 340 nm, and the decrease of this molecule reflects the enzyme activity.

PfATP6 has been reported to be more stable in the detergent Octaethylene glycol monododecyl ether ($C_{12}E_8$) with a supplementation of the lipid 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). Sometimes, when we measure a Ca^{2+}-dependent ATPase activity of a non solubilized protein, we use a calcium ionophore Calcimycin (A23187), in order to avoid the accumulation of calcium in the lipid vesicles.

To reduce the quantity of protein used when measuring the ATPase activity of PfATP6, we developed an activity measurement test in 96 well microplates. This test measures the inorganic phosphate (Pi) liberated during the consumption of ATP by the active protein. This test is also especially practical to test several concentrations of molecules on a small amount of the same sample of protein.

II.10.1 - Coupled enzyme ATPase activity measurement

Classically, 10 µg/ml of protein is used in 2 ml of reaction buffer (50 mM Tes/Tris, pH 7.5; 0.1 M KCl; 1 mM $MgCl_2$; 0.3 mM NADH; 1 mM PEP; 0.1 mg/ml LDH; 0.1 mg/ml PK; 0.05 mM Ca^{2+}; 5 mM MgATP; 0.2:0.05 mg/ml $C_{12}E_8$/DOPC or 0.5 µg/ml of A23187). The reaction undergoes at 25ºC and is started by the addition of the enzyme to the medium and stopped by the addition of a final concentration of 2x600 µM EGTA. The difference between the slopes obtained before and after the addition of EGTA is considered to be due to the Ca^{2+}-dependent ATPase activity. To obtain the specific activity, the concentration of PfATP6 was determined from Coomassie Blue-stained gels, using known concentrations of SR, after SDS-PAGE (section *II.*9).

II.10.2 - Pi liberation ATPase activity measurement

The reaction was performed in 40 µl of reaction mix (2 mM MOPS-Tris (pH 7); 44 mM Tes-Tris (pH 7.5); 90 mM KCl; 2% glycerol; 0.9 mM $MgCl_2$; 0.1 mM $CaCl_2$; 5 mM MgATP; 0.05 mM β-Mercaptoethanol; 2.5% DMSO), for PfATP6 2: 0.5 mg/ml $C_{12}E_8$/DOPC is added, and for SERCA1a 0.5 µg/ml of A23187. The reaction undergoes at 30 ºC and is started by the addition of ATP. For compounds testing, 2µl of each compound dilution is added (or 2.5 % of DMSO for negative control). When mentioned, EGTA is added at 3.75 mM as an inhibition of the Ca^{2+} - dependent ATPase activity. The reaction is performed by first incubating the buffer (50mM Tes-Tris, pH 7.5; 0.1M KCl; 1mM $MgCl_2$) with the $C_{12}E_8$/DOPC on ice. Then the diluted compound (or DMSO) is added, than PfATP6 and finally the MgATP and the tube is immediately incubated at 30°C. Each reaction is initiated 1 – 2 minutes after the precedent one. At each chosen time point (for instance 0, 15, 30 or 60 minutes) 33µl of the reaction is taken, from the corresponding kinetic tube, and stopped by the addition of 17 µl of 10 % SDS. The defined controls (no enzyme, PfATP6 + EGTA) are also treated in the same way.

mM Pi	Volume of K_2HPO_4/KH_2PO_4 0.5 mM solution	Volume of buffer (50mM Tes/Tris, pH 7.5; 0.1M KCl; 1mM $MgCl_2$)
0	0 µl	33 µl
0.05	3.3 µl	29.7 µl
0.1	6.6 µl	26.4 µl
0.2	13.2 µl	19.8 µl
0.3	19.8 µl	13.2 µl
0.4	26.4 µl	6.6 µl
0.5	33 µl	0 µl

Table 14 – Volumes of each solution needed to prepare the Pi range of known concentrations:

A coloring solution of 35 mM ammonium molybdate in 15 mM zinc acetate pH 5 is added to a 10 % ascorbic acid pH 5 solution, at a ratio of 1:4, respectively. After the end of all the reactions, each time point of the kinetic of the ATPase activity is revealed by incubating with 200 µl of the coloring solution, at room temperature for 35 minutes.

A range of known concentration of Pi is done using a 0.1 M potassium phosphate pH 7.4 solution. The Pi solution is prepared from a 0.5 M KH_2PO_4 solution and a 0.5 M K_2HPO_4 solution. The 0.5 M potassium phosphate solution is performed by adding 35 ml of the K_2HPO_4 0.5 M solution and 7.5 ml of the KH_2PO_4 0.5 M solution. The pH is adjusted to 7.4 by adding K_2HPO_4 0.5 M (if pH lower than 7.4) or KH_2PO_4 0.5 M (if pH higher than 7.4). Then the 0.5 mM potassium phosphate pH 7.4 solution is done from the 0.5 M potassium phosphate solution prepared above. The Pi range of known concentration is performed according to Table 14. Each point of the range of the ATPase activity is incubated with 200 µl of the coloring solution, at room temperature for 35 minutes.

200 µl of each point was deposited in a well of a 96 well microplate. The absorbances were measured on a spectrophotometer at 850 nm. The absorbances measured are reported to the values of the range of known Pi concentrations and the liberated Pi during the enzymatic reaction can be quantified. Like this we obtain a specific ATPase activity measurement of our protein.

II.12 - Expression of PfAdT in E. coli

For functional studies of PfAdT we aimed to express this protein in *E. coli.* PfAdT is an ATP/ADP carrier localized on the mitochondria membrane. To measure the ATP transport, a reconstitution in a proteoliposome will be needed. Using *E. coli,* PfAdT will be addressed to the outer membrane when the fused Maltose Binding Protein (MBP) will be exported to the bacteria periplasm. To express PfAdT, we chose to use a C43 (DE3) *E. coli* strain.

II.12.1 - The C43 (DE3) *E. coli* strain

The C43 (DE3) *E. coli* strain was first described by Miroux and Walker in 1996 to be appropriate for membrane protein over-production using pET related plasmids, without toxic effect for *E. coli.* The genotype of this strain is F^- *ompT gal dcm* $hsdS_B(r_B^- m_B^-)$*(DE3)*. F^- - self-transmissible, low-copy plasmid that may contain a resistance marker to allow maintenance and will often carry lac repressor gene and a blue/white screening of recombinants mechanism; *gal* – blocks the bacteria ability to use galactose, cells cannot grow on media that contains galactose as the sole carbon source; deficiency in the *ompT* protease results in higher yields of intact heterologously expressed recombinant proteins; *dcm* - useful to propagate DNA for cleavage with certain restriction enzymes; $hsdS_B(r_B^- m_B^-)$ - mutations in the system of methylation and restriction that allows cloning of DNA and transformation of PCR products without cleavage by endogenous restriction endonucleases; DE3 - is a λ prophage carrying the T7 RNA polymerase gene and lac repressor.

II.12.2 -Transformation of C43 (DE3) *E. coli* strain

The verified vector constructions *PfAdT* wt – pET20b and *PfAdT* K24I – pET20b have been transformed into C43 (DE3) *E. coli* strain, under an ampicillin selection marker. Competent *E. coli* (section *I.3.4*) are unfrozen and 100 µl are added to 100 ng of the vector construction, 7.5 µl of buffer (500 mM $CaCl_2$, 50mM Tris pH 8), Milli Q water *qsp* 200 µl and left for 30 minutes on ice. Heat shock is done for 1 minute at 42ºC, and 1 ml of Lysogeny Broth (LB) medium is added for bacteria growth at 37ºC, 180 rpm during 1 hour. After bacteria are centrifuged and plated into LB – agar- ampicillin medium, overnight at 37ºC or for 2 days at room temperature.

II.12.3 - Expression of PfAdT in C43 (DE3*) E. coli* strain

Following the previous publications on the expression of PfAdT in *E. coli* for functional tests (Razakantoanina et al., 2008), we here describe an adaptation of the protocol. The expression of PfAdT wt and K24I was achieved with vector pET20b, under the control of an IPTG - induced T7 promoter, and a N-terminal MBP fused sequence.

After C43 (DE3) *E. coli* strain transformation (see above for protocol), bacteria are grown in 5 ml of LB – ampiciline medium until reaching an OD_{600} of 0.6 – 0.8 (exponential phase). Induction was initiated by the addition of 1 mM of IPTG, and the expression was undertaken during 2 hours (as described in (Razakantoanina et al., 2008) at 37ºC and 180 rpm. Then the bacteria were harvested at a final concentration of 0.5 OD_{600}, centrifuged at 10.000 rpm (rotor SX4400, Beckman Coulter™ - Allegra X-30R Centrifuge) for 5 minutes. To evaluate the expression of the protein of interest, an aliquote was re-suspended in 20 µl of Milli Q water and 20 µl of D2XU, at a final concentration of 0.01 OD_{600}/µl, and frozen in liquid nitrogen. PfAdT expression was then determined by western blot, using an anti-MBP monoclonal antibody (New England Biolabs) report to Table *13* and section *II.9.3* for experimental procedure.

III – Cytotoxic effect of PfATP6 inhibitors

III.1 – Cytotoxicity Measurement by MTT assay

The cytotoxicity of PfATP6 inhibitors for mammalian cells was determined using Vero cells (kidney epithelial cells extracted from an African green monkey *Chlorocebus sp.*). Vero cells are cultured in RPMI [20]+ 10% FBS [21] at 37ºC and 5% CO_2. When confluence is reached, medium is extracted, the adherent cells are washed in steril PBS - 1X, and treated with Trypsin 1X. Cells are left ~5 minutes at 37ºC and 5% CO_2 until they are completely detached from the flask. The reaction is stopped by the addition of medium supplemented with FBS. Cells are then counted in a hemocytometer. Cells are diluted in RPMI + 10% FBS at a final concentration of 10^4 cells/ml and 100µl is seeded to each well of a 96-well microplate. After 24h at 37ºC and 5% CO_2, cells are adherent to the microplate, the medium can be removed. Serial dilutions of each compound is made first in DMSO, 2µl of each dilution is diluted in 200µl of RPMI + 10% FBS (final concentration of 1% of DMSO) and 100µl is applied to each well of each compound to test. After 48h of treatment with the inhibitor at 37ºC and 5% CO_2, the medium is removed and replaced by 100µl of RPMI + 10% FBS, 20µl of the yellow solution of MTT [22] 5mg/ml in PBS is added to each well and incubated for 4h at 37ºC and 5% CO_2. The medium is removed and the purple crystals formed are dissolved with 10 % SDS and 10mM HCl. The absorbances are red at an OD of 540 nm. Dose-response curves can then be drawn and IC_{50} for each compound determined.

IV – SERCA1a, SERCA1a-E255L and PfATP6 Expression in *Xenopus laevis* oocytes membranes and activity measurement.

The protocols used in this section were adapted from (East, 1994; Eckstein-Ludwig et al., 2003; Geering et al., 1989; Krishna et al., 2001; Woodrow et al., 1999).

[20] RPMI – Roswell Park Memorial Institute medium
[21] FBS – Fetal Bovine Serum
[22] MTT colorimetric assay – MTT (3-(4,5-dimethylthiazol-2-yl)2,-diphenyltetrazolium) is yellow when in water based solution. It is reduced by the NAD(P)H-dependent cellular oxidoreductase mitochondrial enzyme, in living cells, to insoluble formazan purple crystals. These crystals can be dissolved and red at 500 – 600 nm. The absorbance measured is directly proportional to cell growth.

IV.1 – Oocytes Expression Vector Construction

The genes of SERCA1a, SERCA1a –E255L and PfATP6 were cloned into a pXOON vector by Bertrand Arnou. The genes were generated by PCR. PCR products were verified on a 1% agarose gel and purified by the Fermentas GeneJET PCR Purification Kit. The DNA is inserted with the restriction enzymes NotI Fast Digest (Fermentas) and Hind III Fast Digest (Fermentas) into pXOON vector. After the vector construction was verified by sequencing, we can proceed to RNA *in vitro* synthesis.

IV.2 - Expression of SERCA and PfATP6 in X. laevis Oocytes

To express a protein in *Xenopus* oocytes, we need to inject cRNA (complementary RNA) into the animal pole of the oocytes. If we aimed to inject directly the vector construction, we would need to inject into the oocyte nucleus, which is a very difficult task. For this, we perform cRNA *in vitro* synthesis.

IV.2.1 - Phenol-Chloroform DNA extraction

First the DNA is linearized by Nhe I Fast Digest restriction enzyme (Fermentas), for 45min at 37ºC. Then, a phenol-chloroform extraction is performed. DOPC-Nuclease free water is added to the digestion *qs* 200 µl, and 200 µl of phenol is added. After vortexing and spinning for 3 min at 10.000 rpm and 4ºC, the supernatant is removed and 200 µl of chloroform are added. After vortexing and spinning for another 3 min at 10.000 rpm and 4ºC, the supernatant is removed and 1/10 of the final volume of NaAcetate 3 M and 2.5X of 75% ethanol are added. After vortexing the tube is kept at -80ºC for 1 hour. The tube is centrifuged for 30 min at 10.000 rpm and 4ºC, the supernatant is removed and 750 µl of 100% ethanol is added. After vortexing and spinning for 30 min at 10.000 rpm and 4ºC, the supernatant is removed and the pellet is dried in a Vacuum Concentrator at 40ºC for 10 min. The pellet is ressuspended in 8 µl of DOPC Nuclease-free water. The quantity of DNA is measured at the nanodrop.

IV.2.2 – RNA *in vitro* synthesis

After the DNA extraction and precipitation, the kit mMESSAGE mMACHINE T7 Ultra is used for cRNA synthesis.

Capped transcription reaction - To the precedent DNA tube is added in the following order: 2.5 µl T7 2X NTP/ARCA, 0.5 µl 10 X T7 Reaction Buffer, and 0.5 µl T7 Enzyme Mix. The reaction tube is gently mixed and incubated for 2h at 37ºC. 0.25 µl of TURBO DNase is added to the reaction and incubated for 15 minutes more at 37ºC.

PolyA tailing – the reagents were added to the precedent tube in the following order: 9 µl Nuclease-free water DOPC, 5 µl 5X E-PAP Buffer, 2.5 µl of 25 mM $MnCl_2$, 2.5 µl ATP Solution, 1 µl E-PAP. The reaction tube is gently mixed and incubated for 45 min at 37ºC.

Lithium chloride precipitation - The reaction is stopped by adding 12 µl LiCl Precipitation solution. After gently mixing, the tube is kept at -20ºC overnight. The day after, the tube is centrifuged for 15 min at 4ºC at 10.000 rpm to pellet the RNA. The supernatant is carefully removed. The pellet is washed once with 1 ml of 70% ethanol and centrifuged at 10.000 rpm for 15 min at 4ºC. The supernatant is removed and the RNA pellet is re-suspended in DOPC Nuclease-free water. The RNA is quantified in a nanodrop and stored at -80ºC.

IV.2.3 – *Xenopus* oocytes preparation

An adult female frog (*Xenopus laevis*) is anesthetized by immersion in 0.03% benzocaine water. The frog's abdomen is iced for 20 min and the frog is surgically operated to remove the oocytes from the ovary. The frog's skin is sutured and the frog is left to recuperate from anesthesia in shallow water. The oocytes are placed in a OR2 pH 7.2 buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, 5 mM HEPES, 1mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 g/l Polyvinyl, 2.5 mM Pyrolidone, Pyruvic acid, 100 u./ml Penicillin, 100 µg/ml Streptomycin). The oocytes are defolliculated by collagenase treatment: 1ml of collagenase type II (2 mg/ml) in Ca^{2+} free OR-2, and up-and-down through a Pasteur pipette. The oocytes are incubated for 1h at room temperature with gentle agitation. The collagenase solution is removed and a fresh solution is added for another hour. The oocytes are then washed 4 times in fresh OR-2 with Ca^{2+} and incubated for a few hours to overnight prior to injection.

IV.2.4 – Microinjection of the RNA into the *Xenopus* oocytes

The synthetized cRNA is manually injected in each individual oocyte with a Nanoject microinjector. As oocytes are not transparent we need (a) 1) dissecting microscope, 2) cold light source power supply, 3) cold light source goose-necks, 4) microinjectors are mounted on a coarse micromanipulator, and 5) switch pedal and (b) Oocyte injection chamber with microchip sockets for holding oocytes during injection (Figure 58). Glass needles for injection are made from glass capillary with a microelectrode puller from Sutter Instruments, the needle tip is then broken slantwise with dissecting forceps. Needles are filled with oil until half volume. Avoiding air introduction, the cRNA preparations are aspired in the needle. Each injection was made with a settled volume of 46.5 nl of 5 - 30ng of cDNA preparation per injection (Krishna et al 2001), that is gently injected into the animal pole of the oocyte. Oocytes are incubated in OR2 buffer with $CaCl_2$ at 19°C for 2 – 5 days and the solution was daily changed.

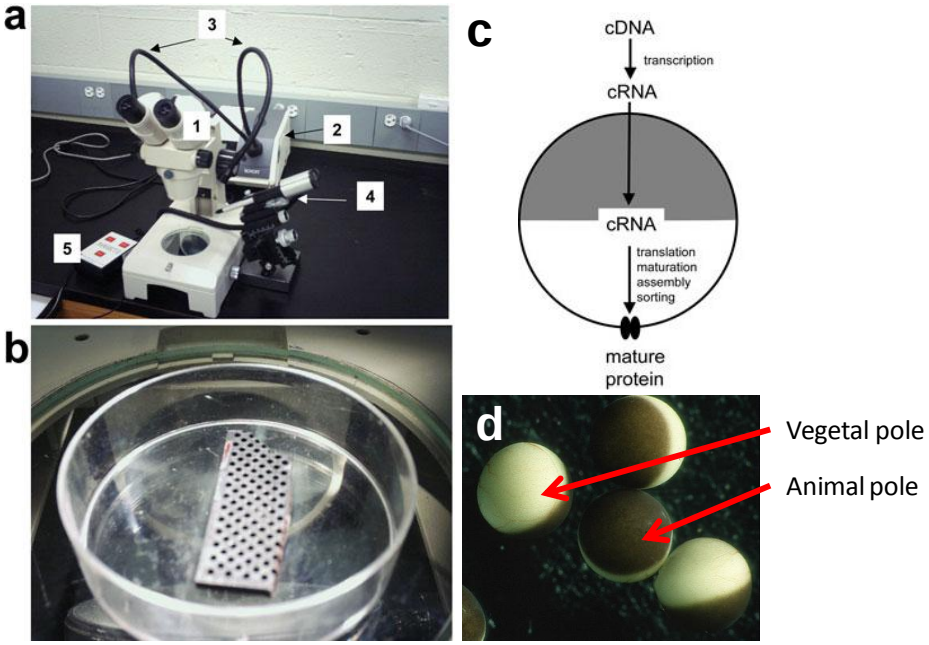

Figure 58 - Oocytes microinjection workstation.

a) *workstation:* ***1)*** *dissecting microscope,* ***2)*** *cold light source power supply,* ***3)*** *cold light source goose-necks,* ***4)*** *microinjector on a coarse micromanipulator, and* ***5)*** *switch pedal;* ***b)*** *injection chamber,* ***c)*** *schematic representation of protein expression in oocytes from cRNA synthesis,* ***d)*** *X. laevis oocytes. adapted from http://www.wormbook.org/.*

IV.2.5 – membrane preparation from injected oocytes

At this stage of the protocol, we tried two different protocols: the one described by S. Krishna and co-workers (Eckstein-Ludwig 2003, Uhlemmann 2005, Krishna 2001); and another protocol from Geering et al. (Geering et al., 1989), that was mentioned by S. Krishna to be used with "minor modifications" in his articles.

Geering protocol - Oocytes are homogenized by 20 up and downs with a P-1000 in buffer A (25 µl/oocyte) containing 83 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.9, and an antiprotease tablet). The homogenates were centrifuged at 1000 g for 5 min at 4°C. The lipids floating on the surface were removed with a cotton stick. The resulting supernatant S1 was centrifuged at 10.000 g for 20 min at 4°C. The resulting supernatant S2 was centrifuged at 100.000 g for 90 min at 4°C. The pellet P3 was re-suspended in equal volume of membranes with HBS (150 mM NaCl, 25 mM Hepes, pH 7.5). The membranes were frozen in liquid nitrogen and kept at -80°C.

Krishna protocol – the same as above although the intermediate centrifugation was replaced by a second centrifugation at 1000 g for 20min and 4ºC.

The expression of SERCA1a, SERCA1a-E255L and PfATP6 was then verified by western blot with specific antibodies (section *II.3.3* and Table 13). Total protein quantification was measured by BCA assay (see section *II.6 - Estimation of protein quantity in P3 membranes*).

IV.3 – ATPase activity measurement of SERCA and PfATP6 and the effect of Artemisinin

The ATPase activity measurement was performed as described in (East, 1994; Eckstein-Ludwig et al., 2003). Report to section *II.10.1 - Coupled enzyme ATPase activity measurement* for experimental procedures. We here used a coupled enzyme based ATPase activity measurement with 100 µg/ml of total protein (200 µg/ml of total protein was also tested for PfATP6), in the conditions above described but in presence of 0.5 µg/ml of A23187. The establishment of a protocol was performed with membranes from oocytes injected with water and rabbit Sarcoplasmic Reticulum (SR) containing SERCA1a. Artemisinin (Sigma-Aldrich) was used at 50 µM, and Thapsigargin (Sigma-Aldrich) was used at 1.5 µM. The Ca^{2+} - dependent ATPase activity was stopped by addition of 1.2 mM of EGTA.

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“Never, never, never give up.”
Winston Churchill

“O que tem de ser tem muita força”
Portuguese proverb

Abstract - New drug discovery for malaria treatment urges, now more than ever. There is no optimal solution to the search for new antimalarials. Worldwide, researchers have focused their energies on several strategies. The most commonly employed are: either by screening molecules issued from chemical libraries in a phenotypic way (i.e., direct testing of drugs on in vitro parasite cultures), or by searching for new molecules acting upon the activity of a specific essential target or pathway. This PhD thesis centers on the second type of approach. We are interested in targeting membrane transporters of P. falciparum. For this, we plan to express proteins of interest in yeast and proceed to their isolation. With optimized functional tests, we aim to: a) Determine the effect of molecules upon specific targets; b) Test their effect on P. falciparum in vitro erythrocytes cultures; c) As well as verify their toxicity on mammalian cells; and d) Perform in vivo testing of the best molecules on a rodent model for malaria. Our actual work is focused on the P. falciparum Ca2+ - ATPase 6 (PfATP6) and adenylate translocase (PfAdT), two essential membrane proteins localized on the endoplasmic-reticulum and the mitochondrial membrane, respectively. We were able to express heterologously PfATP6 in yeast membranes, purify the protein and measure a specific ATPase activity. With this, we have tested a large chemical library and identified specific inhibitors. These were then tested for their effect on in vitro blood stages of P. falciparum and for their cytotoxicity on mammalian cells. For the ATP/ADP carrier PfAdT, we proceeded as previously done with PfATP6 but we have also chosen another type of functional test where we express directly this protein in the plasma membrane of E. coli. This will enable in the future the measurement of radiolabelled ATP uptake, and the identification of specific inhibitors that could then be tested for their effect on P. falciparum in vitro cultures and for their cytotoxicity.

Résumé - La découverte de nouveaux agents antipaludiques est primordiale. A travers le monde, les chercheurs se sont focalisés sur plusieurs stratégies. Les plus développées sont : soit les tests de molécules issues de bibliothèques chimiques dans une recherche phénotypique (comme le test direct d'agents sur des cultures de parasites *in vitro*), soit la recherche de nouvelles molécules agissant sur l'activité d'une cible ou d'une voie spécifique et essentielle. Cette thèse est centrée sur le second type d'approche. Nous nous sommes intéressés aux transporteurs membranaires de *P. falciparum*. Pour cela, nous exprimons les protéines d'intérêt dans la levure et nous les purifions. Nous optimisons les tests fonctionnels, dans le but de : a) déterminer l'effet des molécules sur les cibles spécifiques ; b) tester leur effet sur les cultures d'érythrocytes infectés par *P. falciparum in vitro* ; c) vérifier leur toxicité sur des cellules de mammifères ; et d) réaliser le test des molécules les plus efficaces *in vivo* dans un modèle de paludisme murin. Notre travail actuel est focalisé sur l'ATPase6 de *P. falciparum* (PfATP6) et l'adénylate translocase (PfAdT), deux protéines membranaires essentielles localisées respectivement sur le réticulum endoplasmique et la membrane mitochondriale. Nous exprimons de manière hétérologue PfATP6 dans les membranes de levure, nous purifions la protéine et mesurons une activité ATPase spécifique. Nous avons ainsi pu tester une bibliothèque chimique importante et identifier des inhibiteurs spécifiques. Ces derniers ont ensuite été testés pour évaluer leur effet sur les stades érythrocytaires du parasite *in vitro* et leur cytotoxicité sur des cellules de mammifères. Pour le transporteur PfAdT, nous procédons comme pour PfATP6, mais nous avons choisi un autre type de test fonctionnel dans lequel la protéine est directement exprimée sur la membrane plasmique d'*E. coli*. Cela devrait permettre de mesurer le transport d'ATP radiomarqué, et l'identification d'inhibiteurs spécifiques dont les effets pourront être évalués sur des cultures de parasites *in vitro* et dans des essais de cytotoxicité.